



THE UNIVERSITY OF QUEENSLAND
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**Bovine Genital Campylobacteriosis: Isolation, identification and
virulence profiling of *Campylobacter fetus* subsp. *venerealis* in a small
animal model**

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Abstract

Bovine Genital Campylobacteriosis is a significant disease in Australian beef and dairy herds. The causative organism, *Campylobacter fetus* subsp. *venerealis* is a microaerophilic bacterium that often resides asymptotically in bull penile sheaths and is passed on to females during mating. Virulent strains of the bacterium can cause subfertility and late term abortions in infected females. It has the potential to cause large economic losses and is often difficult to diagnose by culture due to the slow-growing, fastidious bacterium becoming overgrown by contaminants. The gold standard for detection is culture and biochemical testing for identification.

Isolation and identification methodologies for *C. fetus* subsp. *venerealis* have not been reviewed for many years partly due to the lack of an Australian culture collection. Determining the variation in transport media viability, culture phenotyping, addition of newer phenotypic tests or the evaluation of strain virulence relies on the availability of cultures for analysis. Therefore, the objective of this study was to use a shared culture collection to identify best practice for transport media and isolation methodologies, define the phenotypic profile through refined, standardised methods and develop a pregnant guinea pig model to facilitate future studies of the pathogenicity, abortion mechanism and vaccine efficacy of *C. fetus* subsp. *venerealis*.

This study biochemically characterised 152 *Campylobacter*-like isolates to determine the breadth of variability, and to test innovative diagnostic tools for phenotypic characterisations. Strains of *C. fetus* subsp. *venerealis* were used to determine viability in the commonly recommended Lander's transport and enrichment medium (TEM) as well as the recently described Thomann transport and enrichment (TTE) medium *in vitro* and *in vivo*. The *in vitro* experiment also aimed to determine the efficacy of the two transport media with subsequent sub-culture onto: sheep blood agar (SBA) with and without filtration (0.45 µm) and Campylobacter selective agar. Incubation temperature effect was also studied, by comparing TEM enrichment at 25°C and 37°C. The *in vivo* cattle field trial found no significant difference in the isolation of *C. fetus* subsp. *venerealis* between Lander's and TTE ($p=0.1037$), while a higher number of thermophilic *Campylobacter* cultures were isolated from TTE media. *In vitro*, both TTE and Lander's media were able to sustain *C. fetus* subsp. *venerealis* growth for up to 7 days at either 25°C or 37°C, and allow enrichment in spiked mixed cultures with either *P. vulgaris* or *C. jejuni*. Campylobacter selective agar appeared to produce the most consistent positive results from spiked mixed cultures, while SBA was overgrown in most instances and 0.45 µm filtration produced inconsistent results and required a high starting concentration (10^5 CFU/ml). Therefore, diagnostically the use of either TTE or Lander's TEM for transport of samples

followed by plating onto *Campylobacter* selective agar was optimal for isolation of *C. fetus* subsp. *venerealis* from mixed cultures.

All cultures were phenotypically profiled with a selection of strains profiled using 27 different antibiotics. A subset of strains across different *Campylobacter* species were characterised using API Campy kits, Biolog GEN III and Biolog Phenotypic MicroArrays. It was found that automated technologies did not provide any identification advantages over culture phenotyping. API kits were unable to distinguish between *Campylobacter* species, while Biolog PMs had poor reproducibility. The GEN III microplate identified methyl pyruvate as a possible alternative source for H₂S testing for the distinction of *C. fetus* subspecies biovars. Antibiotic profiling identified six antibiotics including ampicillin, cefuroxime, ceftiofur, cephalothin, novobiocin and penicillin which may differentiate between *C. fetus* subspecies, however further research is warranted to confirm these.

A pregnant guinea pig model was developed by testing dose (10^4 , 10^7 and 10^9 CFU/ml), time to abortion (up to 168 h and 288 h), route of inoculation (intraperitoneal vs. intravaginal), and outcomes (culture, PCR and histology) using 7 groups (n=35) of pregnant dams. It identified suitable parameters for inoculation with reproducible results achieved via the intraperitoneal route with a dose of 10^7 CFU/ml to guinea pigs at 5-6 weeks of gestation. Animals were observed for up to 288 h before sampling of dams and fetuses for culture, PCR and histological analysis of placental and uterine tissue. A virulence variation study testing four *C. fetus* subsp. *venerealis* strains in groups of 10 guinea pigs (n=40) was then conducted with a protective dose of an experimental vaccine against bovine campylobacteriosis (determined in a pilot study) as a parameter for strain virulence. The study showed varying levels of virulence across strains, with between 17 – 100% abortions in non-vaccinated groups, 0 – 50% abortions in vaccinated. Histopathology showed placentitis, endometritis and vasculitis of varying severity correlating to time to abortion. Intraperitoneal administration of *C. fetus* subsp. *venerealis* to pregnant guinea pigs is a promising model for the investigation of *C. fetus* subsp. *venerealis* strain virulence variation, abortion mechanisms and potential vaccine efficacy studies.

This study has shown the level of biochemical variation and reliability of these test results for Australian *Campylobacter* isolates as well as optimal transport, isolation and identification methodologies for Australian *C. fetus* subsp. *venerealis* isolates. A small animal model for the assessment of *C. fetus* subsp. *venerealis* strains virulence was developed with the potential to be implemented in vaccine development or extrapolated to cattle infection studies.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Material presented in this thesis in Chapter 6 has been published in the following manuscript:

***Koya A.**, de Wet S.C., Turner S., Cawdell-Smith J., Venus B., Greer R.M., Lew-Tabor A.E, Boe-Hansen G.B., Evaluation and histological examination of a *Campylobacter fetus* subsp. *venerealis* small animal infection model (2015) Research in Veterinary Science, 99: 1-9

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subspecies *venerealis* in cattle. The 6th International Veterinary Vaccines and Diagnostics Conference, IVVDC2012 (Cairns, Australia), poster presentation (abstract 76).

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Contributions by others to the thesis

Dr Gry Boe-Hansen: Primary supervisor and CI of the ARC Linkage project, contributing to the design and execution of the animal models as well as inoculation and sampling of cattle in *in vivo* model. Primarily responsible for critical revision of thesis and publications.

A/Prof Ala Lew-Tabor: Supervisor and CI of the ARC Linkage project, overseeing molecular and microbiological research and review of thesis and publications; Primary PhD supervisor for Dr Indjein's PhD, Ms Nordin and DAF technical staff (Bronwyn Venus) undertaking molecular assay screening.

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List of Abbreviations

°C – Degrees Celsius

µg – Microgram

µL – Microlitre

µm – Micromolar

AF – Amniotic Fluid

AFLP – Amplified Fragment Length Polymorphism

ANZSDP – Australia and New Zealand Standard Diagnostic Procedure

API – Analytical Profile Index

AQIS – Australian Quarantine Inspection Services

ATCC – American Type Culture Collection

BGC – Bovine Genital Campylobacteriosis

BHI – Brain Heart Infusion

CAP – Chorio-allantoic Placanta

CFU – Colony Forming Units

CO₂ – Carbon Dioxide

DAFF – Department of Agriculture, Fisheries and Forestry

DNA – Deoxyribonucleic Acid

DPI – Department of Primary Industries

ELISA – Enzyme Linked Immunosorbent Assay

EM – Endometrium

FAT – Fluorescence Antibody Test

FSC – Foetal Stomach Contents

g – gram

h – hour(s)

H₂O - Water

H₂S – Hydrogen Sulphide

HCl – Hydrochloric Acid

IFAT – Immunofluorescence Antibody Test

IP – Intraperitoneal

IQR – Interquartile Range

IVA – Intravaginal

JZ – Junctional Zone

L – Litre
M - Molar
Mb – Mega Base pairs
mg – Milligram
min – minute(s)
mL - Millilitre
MM – Myometrium
N – Nitrogen
n – number
NaCl – Sodium Chloride
NaOH – Sodium Hydroxide
NCTC – National Collection of Type Cultures
NE – Neutrophil Exudate
NSW – New South Wales
O₂ – Oxygen
OIE – Office International des Epizooties (World Organisation for Animal Health)
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PFGE – Pulse Field Gel Electrophoresis
PM – Phenotypic Microarray
QLD – Queensland
s – second(s)
SBA – Sheep Blood Agar
SD – Standard Deviation
SM – Submucosa
sp. – species
subsp. – subspecies
T4S – Type IV Secretion system
TEM – Transport and Enrichment Media
TSI – Triple Sugar Iron
TTE – Thomann Transport and Enrichment media
UK – United Kingdom
VIC – Victoria

Chapter 1. Literature Review

1.1 Introduction

Bovine genital campylobacteriosis (BGC) is a venereal disease affecting all major cattle farming countries worldwide. It is caused by a fastidious microaerophilic bacteria, *Campylobacter fetus* subsp. *venerealis* which is often asymptotically carried by bulls within a herd. Pathogenic strains may cause infertility or abortions in pregnant heifers, followed by changes to oestrus cycle affecting subsequent time to pregnancy (Clark 1971). This can cause significant economic impact in farming industries and requires careful and thorough diagnosis and treatment to allow prevention. BGC is a notifiable trade disease with countries requiring certification of disease free status of both cattle and semen for importation (OIE 2012).

Vaccines are available for treatment and control of the disease, though minimal efficacy studies are available to prove ongoing protection. Detection of true infection levels within a herd through routine diagnostic testing is inadequate due to difficulties in laboratory diagnosis. Culture remains the gold standard for diagnosis of BGC, however, the bacteria are often overgrown by contaminants, and are sensitive to fluctuations in atmospheric and temperature conditions (Lander 1990, Monke, Love et al. 2002). Molecular and serological testing are not recommended as diagnostic assays for individual animals with discrepancies seen in PCR results and low sensitivity of the ELISA. Serological herd testing can also only be used in heifers due to the detection of immunoglobulins not present in adequate amounts in bull penile sheaths (OIE 2012).

Identification of optimal bacterial enrichment and isolation methodologies is necessary for definitive diagnosis while characterisation of isolates is imperative to allow correct disease conclusion and treatment. Reliable, optimised animal models are needed for vaccine efficacy studies to allow continuing confidence in current vaccines, as well as allow for potential improvements in vaccine strains. Animal models can also be used to further understanding of infection mechanism and pathogenic profiles of *C. fetus* subsp. *venerealis*.

1.2 Aetiology

1.2.1. *Campylobacteraceae*

The *Campylobacteraceae* family consists of three genera; *Campylobacter*, *Arcobacter* and *Sulfurospirillum*. The two former genera have been shown to be associated with disease in humans and animals while *Sulfurospirillum* consist of environmental bacteria. *Arcobacter* genus was developed to include what were previously believed to be aerotolerant *Campylobacter* strains, but were shown to possess distinctly different DNA-rRNA profiles (Vandamme, Falsen et al. 1991). These strains have been shown to be associated with disease in cattle as well as found in healthy animals (Vandamme, Falsen et al. 1991, Driessche, Houf et al. 2005). The current *Campylobacter* genus includes twenty-three confirmed species, of which 10 are considered of interest in veterinary microbiology (McVey, Kennedy et al. 2013), of these *C. fetus*, *C. hyointestinalis*, *C. sputorum* and *C. jejuni* are commonly found in bovine venereal diagnostic submissions. *C. fetus* and *C. jejuni* have been shown to be the causative agents of sporadic cattle abortions, though *C. jejuni* abortion in cattle is rare (Anderson, Blanchard et al. 1990). *C. hyointestinalis* and *C. sputorum* are considered commensal reproductive and digestive tract bacteria (McVey, Kennedy et al. 2013).

1.2.2. *Campylobacter fetus*

In 1906 an investigation by the British government into a surge in ewe abortion incidences identified what is widely believed to be *C. fetus*, described as: “... comma shaped, but long spirilla forms, apparently consisting of several commas joined end to end, were also present. In hanging drops these organisms were actively motile...” (McFadyean and Stockman 1913).

In 1919 when research was conducted into a bovine venereal disease outbreak in the USA, a similar bacteria was identified. Due to the curved ‘vibrio’ shape of the bacteria, it was named *Vibrio fetus* (Smith and Taylor 1919). The bacteria that these researchers observed was reclassified in 1963 as *Campylobacter fetus* within the new genus *Campylobacter*, Greek for ‘curved rod’ which contained vibrio organisms of veterinary significance. These bacteria were defined based on a low Guanine-Cysteine DNA base composition of 29% – 36% (Sebald and Veron 1963). The taxonomic differentiation of *Campylobacter* species was further redefined in 1973 to discriminate between bacteria within the genus based on their catalase and H₂S biochemical properties (Veron and Chatelain 1973). This differentiation created three sets of main characteristics for speciation: catalase positive, H₂S negative *C. fetus* strains, catalase and H₂S positive including *C. coli* and *C. jejuni* and catalase negative *C. sputorum* strains.

The species *Campylobacter fetus* is further subdivided into three subspecies, *C. fetus* subsp. *testudinum*, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. The former is of reptilian origin (Fitzgerald, Tu et al. 2014) while the latter subspecies consists of two biovars; *Venerealis*, and *Intermedius*. Though no distinguishing pathogenic or epidemiological characteristics have been identified within the biovars, they possess slight variations in biochemical properties. The changing nomenclature and revisions of speciation has been on-going since the identification of the bacteria almost a century ago. Improvements in taxonomic techniques, genomics and increased research into defining characteristics may continue to expand these classifications.

1.2.3. Bacterial Features

C. fetus subsp. *venerealis* are small, curved rod shaped bacteria measuring approximately 0.5 – 5 µm long and 0.2 – 0.3 µm wide (Smith and Taylor 1919, Sebald and Veron 1963). The bacteria are Gram-negative and display a spiral, corkscrew motility due to the single flagella protrusion present at one or both ends (Penner 1988). They are microaerophilic, requiring specific atmospheric conditions of 5% O₂, 10% CO₂, and 85% N₂ at 25°C - 37°C to sustain growth (Veron and Chatelain 1973, On and Holmes 1991). The bacteria are highly fastidious, requiring 48 - 72 hours at ideal atmospheric conditions to produce bacterial colonies on agar, or a cloudy bacterial mass in broth, just below the surface (Smith and Taylor 1919, Hum, Hornitzky et al. 2009). Morphologically, the bacteria present as smooth, pink-grey, shiny, non-haemolytic colonies of 1-3 mm in diameter (OIE 2012).

1.2.4. Biochemical Properties

Bacterium belonging to the genus *Campylobacter* share certain basic biochemical characteristics; Gram negative, oxidase positive and growth microaerobically. At the species level, *C. fetus* is catalase positive and reduces nitrate. The distinguishing test between the two subspecies is growth in 1% glycine medium, with *C. fetus* subsp. *fetus* able to tolerate the substrate, while *C. fetus* subsp. *venerealis* is typically unable to grow in its presence. Research has however shown that glycine tolerance is mediated by phages allowing acquisition or loss of the characteristic, reducing its reliability (Chang and Ogg 1971). Hydrogen sulphide production has been considered a differentiating feature between the two subspecies, with *C. fetus* subsp. *fetus* producing H₂S in the presence of cysteine while *C. fetus* subsp. *venerealis* does not, however, the identification of a biovar of the latter subspecies, *C. fetus* subsp. *venerealis* biovar *Intermedius*, which also produces H₂S, has dismissed the test as a defining feature (OIE 2012).

The current recommended *C. fetus* subsp. *venerealis* characteristics used for biochemical differentiation are stipulated within the OIE guidelines (OIE 2012). The bacteria are asaccharolytic, displaying no fermentation or oxidation of carbohydrate sources (On 1996).

1.2.5. Other Bovine *Campylobacter* species of Significance

The most common species contending with diagnosis of *C. fetus* subsp. *venerealis* is the closely related *C. fetus* subsp. *fetus*. Differentiation between the two subspecies is critical due to the differences in pathology and epidemiology. *C. fetus* subsp. *fetus* more commonly infects sheep, rather than cattle, is responsible for sporadic abortions and embryonic deaths, and is able to colonise both the intestinal and genital tracts (Clark 1971). *C. fetus* subsp. *fetus* also causes infection in humans, with bacteraemia, abortions and gastroenteritis. *C. fetus* subsp. *fetus* is usually ingested by cattle from contaminated surroundings and while ordinarily found in the enteric system, it can occasionally spread venereally producing vaginal infections in susceptible heifers (Irons, Schutte et al. 2004). A survey conducted over 15 months into bovine abortions in Victoria, Australia, received samples from 40 abortive events on 37 farms (Hum 1987). Culture of the causative organism from submitted foetal samples found 31 *C. fetus* subsp. *venerealis* isolates on 28 farms and 9 *C. fetus* subsp. *fetus* isolates on 9 farms (as distinguished by glycine tolerance biochemically). While the former appeared to be more prevalent, there was no symptomatic differences in animals infected, abortion ages or ability to cause multiple deaths per farm. While this does question the purported epidemiological and pathological differences in the bacteria, it also displays the need for good reproduction record keeping for identification of infertility, to definitively identify the symptom as a cause of *C. fetus* subsp. *venerealis* infection only.

Other species isolated from cattle include *C. hyointestinalis* and *C. sputorum* biovar *sputorum*, previously identified as '*V. bubulus*' (On, Atabay et al. 1998). These are typically found in faecal samples, however, '*V. bubulus*' has been shown to be a saprophytic bacteria present in preputial samples (Lovell 1963). These species have not yet been identified as the causative organism of any disease, often being carried asymptotically. Isolations of penile sheath *Campylobacter* organisms during abattoir surveillance found a low prevalence of thermophilic *Campylobacters* (Indjein 2013). These often contended with isolation of *C. fetus* subsp. *venerealis* during laboratory diagnosis.

1.3 Epidemiology

1.3.1. Bovine Genital Campylobacteriosis

Bovine Genital Campylobacteriosis (BGC) is present in many extensive cattle farming countries across most continents, with clinical disease status in a number of them (OIE 2012). These include Australia, North and South America, Europe and Africa (Figure 1). It has also been shown to be present in Canada (Waldner, Hendrick et al. 2013) and Iran (Hosseinzadeh, Kafi et al. 2013, Waldner, Hendrick et al. 2013). A review of the disease epidemiology speculated it to be endemic in developing countries where lack of resources does not permit the diagnosis of the disease and where minimal data is available of on-farm herd health (Mshelia, Amin et al. 2010). Though the OIE database aims to produce a continuous updated profile of the disease distribution, it is only as relevant as the information submitted for inclusion. Clinical disease in developed countries appears to be more common in beef herds where natural service is often used, whereas dairy farms using artificial insemination or controlled, restricted mating are able to greatly reduce the risk of infection (McGowan and Murray 1999).

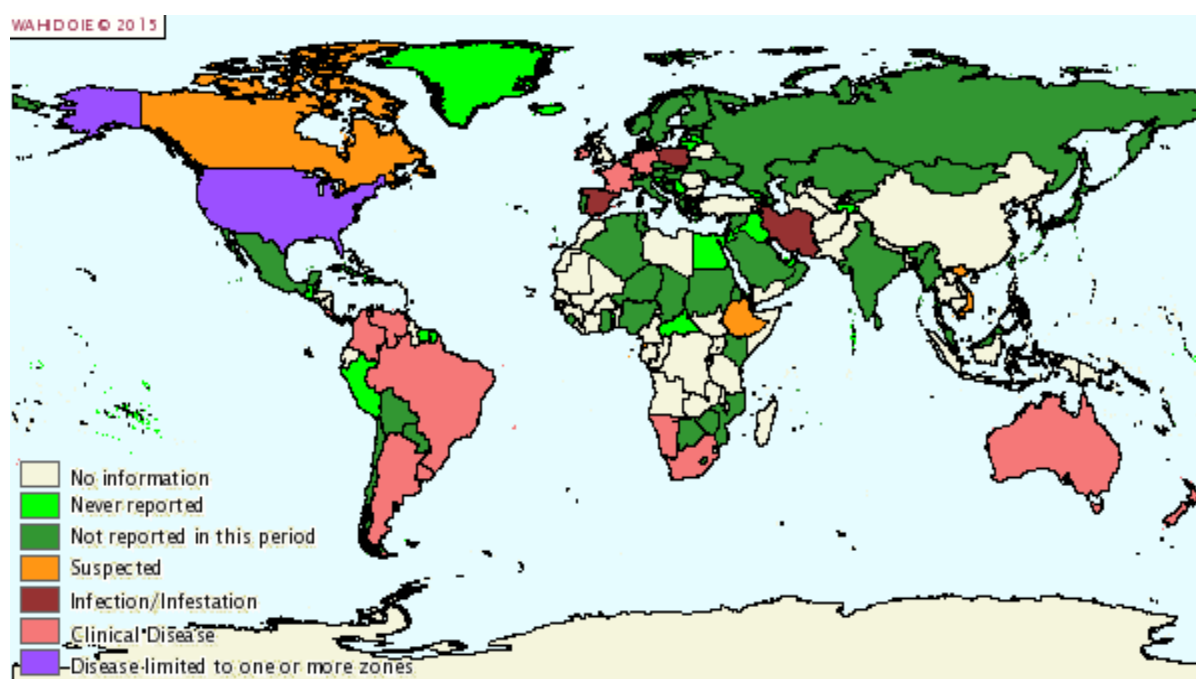


Figure 1. Global distribution of BGC from July to December 2013 adapted from OIE 2015.

The economic impacts of the disease can be significant due to restrictions on international trade of animals and semen from herds with known infection, decreased and delayed calving rates and the necessitation of culling of infected cattle (Vandeplasseche, Florent et al. 1963, Dufty and Vaughan 1993, OIE 2012). It has been suggested to be liable for up to a 60% decrease in calving rates in

newly infected herds (Clark 1971) with 35% decrease in revenue in herds with established infection (McCool, Townsend et al. 1988). Herd fertility rates may improve after 6 months with increased breeding cycles allowing improved pregnancy rates through multiple services (Vandeplasseche, Florent et al. 1963, Dufty and Vaughan 1993). A personal communication supplied by J.R Lawson and D.J MacKinnon in a review of BGC showed that an average of 5.2 inseminations or services was needed to produce 81% pregnancy rates in infected heifers, while non-infected heifers required an average 1.9 services to produce a 99% pregnancy rate (Laing 1956). There has been some evidence of milk production impact in infected dairy herds (Akhtar, Riemann et al. 1993, Akhtar, Riemann et al. 1993) but few studies with minimal evidence are available to elucidate the true impact.

In Australia, incidences of BGC have been considered to be prevalent with studies showing continuous detection over the last 50 years. An 8-year long survey of *Vibrio fetus* infection in beef and dairy herds in New South Wales had high levels of detection using vaginal mucus agglutination (VMA) testing (Jakovljevic and Beattie 1969). Culture correlation was extremely low, with '*V. fetus*' isolated in 0.09% (3/3180) of vaginal mucus samples from dairy cattle with infertility problems, 0.35% (1/288) of bull semen samples, 0% bull preputial washes and 6.6% (16/244) of aborted fetuses. It was noted that there was failure to isolate a causative organism in 75.8% of aborted foetal samples. Comparatively, VMA testing showed a higher incidence, with 51.4% (452/879) of dairy and 52% (66/127) of beef cattle tested, positive. A further 37.3% (275/773) of animals tested at abattoir were found to be positive. The discrepancies in culture to VMA correlation were considered to be inadequacies of culture technique, and delay in reaching the laboratory (Jakovljevic and Beattie 1969).

Testing of subsets of cattle from 41 herds at muster during 1985-1986 in the Northern Territory found 87.8% (36/41) of herds to be infected by fluorescent antibody technique (FAT) (McCool, Townsend et al. 1988). In 1987 a survey of abortion events in New South Wales found 77.5% (31/40) to be caused by *C. fetus* subsp. *venerealis* as determined from histopathology and culture (Hum 1987). A subsequent study into the prevalence of the disease in New South Wales using ELISA, found up to a 34.8% incidence in screened herds (Hum, Quinn et al. 1994). In New Zealand a study using an ELISA detecting IgG in vaginal mucus samples from 125 herds (n=1230) found 70% of tested herds to be positive (McFadden, Heuer et al. 2004). There was no significant difference amongst herds recorded as having low, medium or high fertility rates, with a further 54 bulls from 9 low fertility classified herds all testing negative by culture (McFadden, Heuer et al.

2004). However, other *Campylobacter* species including *C. jejuni* and *C. fetus* subsp. *fetus* were isolated, leading the authors to hypothesize cross-reactivity of the ELISA producing false positives in New Zealand herds (McFadden, Heuer et al. 2004).

1.4 Pathogenesis

1.4.1. Heifers

As stated, the causative bacterium is carried by infected bulls asymptotically, and passed to heifers and cows during natural service. The bacteria is able to colonize the vagina and subsequently pass through the cervix into the uterus where higher concentrations may result in endometritis (Adler 1957). Vaginal infection is only seen in 10-20% of heifers (Clark 1971), with a further 25% displaying infection in oviducts (Vandeplasseche, Florent et al. 1963). Infection in the uterus is frequently associated with mild endometritis with suggestions that the histological inflammation in conjunction with bacterial effects on fertilization or implantation may result in infertility (Vandeplasseche, Florent et al. 1963). Abortions have been speculated to occur in 5-10% of infected heifers at approximately 5 months of gestation (Clark 1971) with a study finding an average of 6 months (4.5 – 8 months) in 40 *C. fetus* abortion events (Hum 1987). The bacteria do not appear to hinder the fertilization or development of the embryo as displayed in *in vitro* studies (Bielanski, Sampath et al. 1994), but it is assumed that the endometritis found in infected heifers provides an inhospitable environment for embryo and foetal survival (Estes, Bryner et al. 1966).

Infection within the uterus stimulates an immunological response, producing IgM, IgA and IgG (Van Aert, De Keyser et al. 1977). Identification of immunoglobulin response in heifers either immunized (systemically or locally), or with cervicovaginal or intrauterine infection found all three immunoglobulins to be present in cervicovaginal mucus (CVM) samples at different times (Corbeil, Duncan et al. 1974). In simulated short term infections (48-50 days), IgM (day 29) and IgA (from day 30) were found in 3 animals (n=4), while IgG was only present in one heifer at day 40. The pattern for response was the same in the longer term infection group, with IgM preceding IgA which preceded IgG. The results from systemically immunized animals showed IgG predominantly present, with highest concentrations in serum and uterine secretions, while in locally immunized animals IgA was predominantly present in CVM. These findings were indicative of IgG being responsible for establishing protective immunity and preventing subsequent colonization, while IgA which immobilizes bacteria rather than killing them, may assist with preventing bacteria passing through the cervix leading to uterine inflammation (Corbeil, Duncan et al. 1974).

Pathologically, heifers show little histological change within the vagina and cervix with gradual lymphocyte and neutrophil infiltration during infection. However, within the uterus, vascular degenerations, hyperplasia, marked neutrophil and lymphocyte aggregation and chronic superficial endometritis are often noted in acute infection (Dozsa, Olson et al. 1960, Estes, Bryner et al. 1966).

1.4.2. Bulls

C. fetus subsp. *venerealis* has a tropism for the genital tracts of cattle and is assumed to be more prevalent and allowing for prolonged infection in older bulls where deeper crypts might make for a more hospitable environment (Bondurant 2005). A study using artificial and natural infection (mating with carrier heifers) showed that *C. fetus* subsp. *venerealis* was able to establish transient infections in 4 bulls (n=5) from 18 months of age, with established infection only possible between 40-70 months of age (Dufty, Clark et al. 1975). A second experiment using 17 bulls in three age categories (20, 34, and 47 months) showed that 76.5% (n=13) of animals were able to become infected; three in 20 month age group, five each in the two remaining groups. Fifty-three percent of the animals established infection under 47 months of age, though infection rate increased with time and exposure (Dufty, Clark et al. 1975). This may not be the best indication of in field infection, where strain variations may have an impact on establishment as well as older bulls potentially having longer contact with infected animals. The bacteria is most concentrated in the fornix of the prepuce, and due to weak antibody response, can survive prolonged periods (Samuelson and Winter 1966, Bier, Hall et al. 1977). A comparison of immunoglobulin levels in 12 bulls of two different age categories (3-4 years; 5-6 years) were monitored. It was found that while older bulls presented with higher levels of immunoglobulins in preputial secretions, the overall relative concentration of immunoglobulins was the same in both groups (Bier, Hall et al. 1977). Pathologically, there appears to be no macroscopic lesions present in the prepuce, while only a low infiltration of lymphocytes and plasma cells was noted in histological analysis of preputial mucosa of infected bulls of different ages (Samuelson and Winter 1966, Bier, Hall et al. 1977).

1.4.3. Treatment and Control

Vaccines for the prevention of BGC are available through different manufacturers in countries possessing large cattle production economies, such as Argentina (Vacuna anti *Campylobacter* 40[®], CDV Lab), Australia (Vibrovax[®], Zoetis), South Africa (Vibrin[®], Pfizer Animal Health; *Vibrio fetus* vaccine, Onderstepoort Biological Products Ltd.) and USA (Vibrin[®], Pfizer Animal Health, New York). Studies carried out in South America have shown varied efficacy in recent years (Cobo, Cipolla et al. 2003). The pregnancy rates, antibody titres and infection period were monitored for 20

eight heifers vaccinated with a commercial vaccine (Bioabortogen-H[®], Biogenesis, Argentina), another eight heifers with a second vaccine (Repropolovac[®], San Jorge Bago, Argentina) and eight control unvaccinated animals. Pregnancy rates and antibody levels were found to be low in all three groups, with 25% and 38% pregnancy rates in the two vaccinated groups, and no animals becoming pregnant in the control unvaccinated group (Cobo, Cipolla et al. 2003). The current Australian vaccine, Vibrovax[®], available from Zoetis, was developed 40 years ago as a bivalent (*C. fetus* subsp. *venerealis* biovar *Venerealis* and *C. fetus* subsp. *venerealis* biovar *Intermedius*) colloidal suspension to be administered (twice, five weeks apart) as a preventative mechanism to herds annually.

Vaccination has also been used successfully as a curative in infected bulls (Foscolo, Pellegrin et al. 2005) and heifers (Eaglesome, Garcia et al. 1986). Twenty-seven bulls positive by direct fluorescent antibody testing (DFAT) were vaccinated twice, 23 days apart with an experimental vaccine. After the first vaccination, 44.5% of bulls tested negative, with a total of 55.6% of animals negative after both vaccinations (Foscolo, Pellegrin et al. 2005). Curative effect in heifers was similarly effective with 44% of vaccinated infected heifers clearing infection, compared to 15% of unvaccinated heifers, tested over 17 weeks (Eaglesome, Garcia et al. 1986). Other farming practices include the use of dihydrostreptomycin intramuscular injections (22 mg/ kg body weight), which have been used concurrently with vaccination in infected bulls on artificial insemination studs successfully (n=17) (Garcia, Ruckerbauer et al. 1983). The use of dihydrostreptomycin or streptomycin are no longer viable options in some countries, including Australia with the aim of curbing antibiotic resistances in food producing animals (NRA 1999).

Additional prevention procedures in use include artificial insemination with certified negative semen or mating with young, virgin bulls, both of which are costly and impractical for extensive farms. Prevention is of higher importance in Australian farms where natural service in large herds is the normal breeding protocol compared to parts of Europe that have less extensive production systems allowing different preventative measures to be taken, including artificial insemination or controlled mating (Bondurant 2005).

As the only symptoms associated with the disease are reduced calving rates due to abortion or infertility, good record keeping on reproduction rates across herds is necessary to allow trends in reproductive health to be determined. A rapid decline in calving, or a spread in heifers calving due

to multiple services required for pregnancy could be indicative of infection and prompt diagnostic testing (Truysers, Luke et al. 2014).

1.5 Diagnosis

The current gold standard for the diagnosis of BGC is isolation of *C. fetus* subsp. *venerealis* using culture, however, considerable time and expertise are required for isolation and identification of the causative bacterium. As the bacteria are of such a fastidious and fragile nature, culture success is often inconsistent allowing possible false negative diagnosis. It is therefore recommended that 4 – 6 negative test results be used to declare a bull disease free, while a single positive sample is indicative of herd infection (Clark 1971). In the development of a real-time PCR method with the comparison of 249 bull smegma samples by culture or PCR, 12.1% were culture negative but PCR positive (McMillen, Fordyce et al. 2006). While there are other factors potentially influencing this result, including low concentrations of viable bacteria, the presence of non-viable bacterial DNA or collection method, a false diagnosis could be detrimental to herd health. The study also tested three known infected bulls multiple times, which were all found to be consistently positive by PCR, with variable culture results dependent on sampling technique used (McMillen, Fordyce et al. 2006). Overgrowth of samples with contaminating organisms can also hinder the diagnosis of an infected animal with transport media and time to laboratory allowing overgrowth (covering 75% - 100% of the agar) in up to 80.6% of samples collected (Monke, Love et al. 2002).

The recognised lower sensitivity of the culture technique due to the fastidiousness of the organism coupled with limitations of isolation methodologies can produce variable results. Studies have been undertaken on improvement and development of transport media for *C. fetus* subsp. *venerealis* (Clark, Monsborough et al. 1974, Lander 1990, Harwood, Thomann et al. 2009, Hum, Hornitzky et al. 2009), as well as identification of optimised isolation techniques (Dufty and McEntee 1969, Hum, Brunner et al. 1994, Monke, Love et al. 2002, Chaban, Guerra et al. 2013, Guerra, Chaban et al. 2014). Molecular detection of the bacteria is often more sensitive than culture, showing a need for improved culture techniques. Concurrent molecular and culture testing of animals found 17% (2/12) PCR positive with all animals culture negative (Chaban, Chu et al. 2012). A second study showed that of 260 samples collected from known infected bulls, 86.9% were culture positive, while 95.8% were positive by Hum PCR (Guerra, Chaban et al. 2014). Further, in the development of the McMillen PCR, a total of 30 PCR positive samples were culture negative (n=249), while 4 culture positive PCR negative samples were shown be nonspecific cultures (McMillen, Fordyce et al. 2006). While numerous PCR assays for identification of *C. fetus* subsp. *venerealis* are apparent

in the literature, they are not routinely used for the diagnosis of BGC independently, but rather complementarily or as a confirmatory test on culture (OIE 2012). Recent studies have demonstrated an ongoing lack of consistency between phenotypic and molecular characterisation, identifying the need for more definitive biochemical testing methodologies (van der Graaf-van Bloois, Miller et al. 2014).

Diagnosis of BGC is made by culture of the causative organism from either vaginal mucus, bull preputial samples and if present, aborted foetal swabs for culture and confirmation by molecular testing (Hum, Quinn et al. 1997, McMillen, Fordyce et al. 2006, Abril, Vilei et al. 2007).

1.5.1. Enrichment and Culture

There have been a number of transport and enrichment media (TEM) used for improved survival and isolation of the bacterium. These include Clarks (Clark, Monsborough et al. 1974), Lander's TEM (Lander 1990) and most recently TTE (Thomann Transport and Enrichment) media (Harwood, Thomann et al. 2009). Due to the very slow growing nature of the bacterium and the potential overgrowth by contaminants, use of an optimum selective media is imperative if samples are not directly delivered to laboratories. The current OIE protocol recommends the use of Lander's TEM for the transport of field isolates for the culture of *C. fetus* subsp. *venerealis* and PBS for molecular testing (OIE, 2008; Lander's, 1990).

1.5.2. Diagnostic Samples

Samples submitted to diagnostic laboratories for culture of *C. fetus* subsp. *venerealis* from bulls include semen or smegma and from heifers, vaginal mucus or aborted tissues if available. These samples may be collected via pipette aspiration, washes, swabbing or scraping. Smegma scrapings, in comparison with preputial washing using sterile peptone water and aspiration with a pipette was shown to be the most successful collection technique in three infected bulls (Tedesco, Errico et al. 1977). Twenty-eight samples were collected weekly by either method and treated equally for culture. Culture results were positive by scraping in 71.4% of collections, washings in 25.8% and aspiration in 36.0% (Tedesco, Errico et al. 1977). Contamination was also shown to be reduced using the scraping method with 33.9% of plates showing high contamination, compared to 83.9% by washing and 63.0% by aspiration (Tedesco, Errico et al. 1977). Preputial washing with phosphate buffered saline (PBS) as used in other studies (Dufty and McEntee 1969, Hum, Hornitzky et al. 2009) while still suitable, may not be the ideal method for collection of samples. It

is however less stressful for the bull compared to pipette aspiration, which was shown to have blood in some collections which could impede molecular testing through inhibition.

The presence of *C. fetus* subsp. *venerealis* in smegma and vaginal mucous collections makes preputial and vaginal scrapings, washings and swabs, all potential samples for successful culture. Limiting exposure of the sample to the surrounding atmosphere may assist growth of the microaerophilic bacteria (Monke, Love et al. 2002). The main sample characteristic affecting culture success as noted by Lander is the initial concentration of *Campylobacter* colonies present in the sample in comparison to contaminants, as well as time from inoculation to incubation of TEM (Lander 1990). The concentration of *C. fetus* subsp. *venerealis* present in preputial samples can vary from 10^2 to 10^5 CFU/ml (Clark 1971), while common contaminants such as *Pseudomonas* sp. and *Proteus* sp. are abundant and fast-growing (Clark 1971, Lander 1990, Lander 1990, Monke, Love et al. 2002).

1.5.3. Enrichment Media

a. Lander's Transport and Enrichment Medium (Lander's TEM)

The initial transport medium predominantly used in Australian laboratories was Clark's, a semi-solid medium with high concentrations of 5-fluoro-uracil as well as polymyxin B sulphate (Clark, Dufty et al. 1974). This media was used successfully in a number of studies, however, Lander noted that it was used with variable success in their laboratory (Lander 1990). Lander's TEM (also known as Weybridge) was developed using components shown to support growth of fastidious, microaerophilic bacteria, such as *C. fetus* subsp. *venerealis*. These include an antibiotics combination developed specifically for the preferential isolation of *Campylobacters* (Skirrow 1977), a supplement to increase aerotolerance of *C. fetus* isolates (George, Hoffman et al. 1978) and charcoal which was shown to support the growth of other fastidious organisms including *Mycobacteria* (Hirsch 1954, Hirsch 1954) and *Leptospira* (Myers, Varela-Díaz et al. 1973). The medium was tested using 21 *C. fetus* strains, 6 *C. jejuni*, *Pseudomonas aeruginosa* and *Proteus vulgaris* inoculated at three different concentrations, 10–31, 100–350 and 10^4 CFU/ml. All TEM tubes were either incubated directly or after 72 h, to simulate transport. It was found that even at the lowest concentration the medium was able to enrich 90.5% (19/21) of *C. fetus* strain incubated directly and 30% (7/21) after 72 h simulated transport. At the second concentration 100% were enriched in direct incubation and 95.2% (20/21) after delayed incubation. The highest concentration allowed enrichment of all species regardless of time to incubation. Fifty percent (3/6) of *C. jejuni* strains were enriched at the highest concentration and none at the two subsequent dilutions. The

contaminants tested were shown to be inhibited at concentrations below 10^3 for *Pseudomonas* and 10^6 for *Proteus*.

Subsequent studies have shown preference for Lander's TEM over Clark's with a comparison identifying 74% positive isolations (14/19) from infected cattle compared with 26% (5/19) from Clark's medium regardless of agar used (Table 1) (Hum, Brunner et al. 1994). Lander's noted in the development of this media that the highest concentrations of antibiotics still allowing multiplication of *C. fetus* was used. This level was determined by using "a few" strains in a preliminary study (Lander 1990) and may not be representative of all *C. fetus* strains. The inoculation of Lander's TEM with filtered preputial samples (passed through a 0.6 μ m filter attached to a syringe) compared with those not filtered found it to be successful in 10.6% of samples; however, 16% of samples were positive from unfiltered inoculum and negative when filtered. This could be due to extremely low initial concentrations of the bacteria and suggest that filtration should be used with caution, or concurrently (Lander 1990). While this study, and the current recommended protocols, suggest incubation of the TEM at 37°C for 72 h prior to plating, a study into contamination relative to time incubated showed that incubation of TEM overnight reduced success of isolation from 45.1% to 27.8% (Monke, Love et al. 2002). Similarly, a study comparing direct plating of samples collected in PBS with overnight incubation of TEM at 25°C to replicate transport, found 86.5% and 35.0% culture success rates, respectively on sheep blood agar (SBA) (Chaban, Guerra et al. 2013).

Table 1. Comparative media success rates for isolation of *C. fetus* subsp. *venerealis* from preputial wash samples collected from bulls and swabs of cervico-vaginal mucus from heifers.

	Saline/PBS*	Weybridge/ Lander's	Clarks	Cary-Blair
Lander (1990)	80.3% (49/61)	90.2% (55/61)	N/A	N/A
Hum, Brunner et al. (1994)	94% (18/19)	74% (14/19)	25% (5/19)	N/A
Monke, Love et al. (2002)	18.1% (13/72)	67.2% (45/67)	N/A	25.4% (18/71)

*saline was directly plated at time of collection, while TEMs were incubated prior to plating; N/A – not applicable

b. Hum's Modified Lander's

A study into antimicrobial resistances of *C. fetus* subsp. *venerealis* isolates found 90% (27/30) of strains to be sensitive to polymyxin B (Jones, Davis et al. 1985). This prompted the modification of Lander's TEM to a reduced concentration of polymyxin B from 10 IU/ml to 2.5 IU/ml (Hum,

Hornitzky et al. 2009). Due to unpublished findings by M. Hornitzky and T. Berg showing sensitivity of *C. fetus* subsp. *venerealis* strains to 5-fluoro-uracil, it was removed from the media (Table 2).

c. Thomann Transport and Enrichment Medium (TTE)

The more recent development of a transport media, Thomann Transport and Enrichment Medium (TTE) aimed to allow the use of the same media for culture and molecular testing. TTE was developed with reduced concentrations of polymyxin B sulphate, trimethoprim and vancomycin. Cyclohexamide and 5-fluoro-uracil were removed in favour of amphotericin B as a fungal inhibitor. The exclusion of charcoal and horse blood from the medium base made it suitable for molecular detection (Table 2). Previous TEMs were only suitable for culture with PBS used for PCR. This necessitated the collection of two samples per animals, and increased time and resources. Comparison of TTE and Lander's TEM identified isolation of *C. fetus* subsp. *venerealis* from TTE for a longer period of time of up to 8 days across three different temperatures; 4°C, 25°C and 37°C while Lander's TEM only allowed isolations for the equivalent time at 4°C. At 25°C and 37°C the bacteria was viable in Lander's TEM for up to five and four days, respectively (Harwood, Thomann et al. 2009). To illustrate the molecular testing capacity of the medium, *C. fetus* subsp. *venerealis* in TTE was spiked with various contaminants including *P. aeruginosa*, *C. sputorum* and *Proteus mirabilis*. These were incubated at 37°C for 72 h, with cultures plated at each 24 h interval. At 72 h DNA extraction and testing using the Abril PCR (Abril, Vilei et al. 2007) was performed, producing positive results in all samples. Culture was ineffective for samples spiked with *P. aeruginosa* and *P. mirabilis* showing the need for complimentary molecular testing, which in this study, could be performed on the single sample (Harwood, Thomann et al. 2009).

Table 2. Comparison of components of three commonly used transport and enrichment media (TEM) for the isolation of *C. fetus* subsp. *venerealis*.

	Lander's Medium (Lander 1990)	Modified Lander's Medium (Hum, Brunner et al. 1994)	Thomann Transport and Enrichment Medium (Harwood, Thomann et al. 2009)
<u>Base Medium</u>			
Mueller-Hinton Broth	22 g/l	22 g/l	Nutrient broth - 25 g/l
Charcoal	0.5% w/v	0.5% w/v	None
Horse blood	7% w/v	7% w/v	None
<u>Antibiotics</u>			
Polymyxin B sulphate	10000 IU/l	2500 IU/l	1875 IU/l
Trimethoprim	20 mg/l	20 mg/l	3.75 mg/l
Vancomycin	40 mg/l	40 mg/l	7.5 mg/l
<u>Fungistat</u>			
Amphotericin B	None	None	5 µg/ml
Cyclohexamide	100 mg/l	100 mg/l	None
5-fluoro-uracil	500 mg/l	None	None
<u>Aerotolerance</u>			
Sodium pyruvate	250 mg/l	250 mg/l	250 mg/l
Sodium metabisulphite	250 mg/l	250 mg/l	250 mg/l
Ferrous sulphate	250 mg/l	250 mg/l	250 mg/l

g – grams; l – litre; w/v – weight per volume; mg – milligram; IU – international units

1.5.4. Agar

After transport and incubation of the media, subculturing onto a selective agar such as Skirrow's, which contains the same selective agents as Lander's medium is recommended (OIE 2012). Non-selective sheep blood agar (SBA) may also be used, in conjunction with a 0.65 µm filter (OIE 2012); this offers size and motility exclusions, whereas selective agar uses antibiotic sensitivity exclusion. Plates are incubated in a microaerobic chamber at 37°C or in specially designed airtight jars with oxygen-reducing sachets in a 37°C incubator. A study of five infected bulls over 10 weeks, sampled twice per week, found up to a 30.6% loss of cultures due to overgrowth of plates (Dufty and McEntee 1969). In one animal 80% (16/20) of samples collected were overgrown with *P. mirabilis*. A comparative study of Lander's TEM using filtered vs. unfiltered collections found that 16% of samples that were negative by culture due to contaminant overgrowth, but were positive from the filtered sample were due to *P. aeruginosa* (Lander 1990). These contaminants are both much larger in size than *C. fetus* subsp. *venerealis* allowing the use of a filter for size exclusion to

reduce overgrowth. The use of antibiotics supplemented agar such as Skirrow's (polymyxin B sulphate, trimethoprim and vancomycin), or *Campylobacter* agar (cyclohexamide, polymyxin B sulphate, rifampicin and trimethoprim) may reduce contaminant growth (Monke, Love et al. 2002, Chaban, Guerra et al. 2013). However, the use of selective antibiotics could preclude the culture of any *Campylobacter* strains that may be sensitive to them.

Table 3. Comparative agar success rates for isolation of *C. fetus* subsp. *venerealis* from preputial wash samples collected from bulls and swabs of cervico-vaginal mucus from heifers.

	Sheep Blood Agar (SBA)	SBA + filter	Skirrow's agar	Campylobacter agar	Greenbriar agar
Hum, Brunner et al. (1994)	N/A	N/A	56.1% (32/57)	64.9% (37/57)	N/A
Monke, Love et al. (2002)	28.6% (20/70)	N/A	46.5% (33/71)	N/A	33.3% (23/69)
Chaban, Guerra et al. (2013)	N/A	35% (43/124) ^a 86.5% (237/274) ^b	40% (50/124) ^{a,c} ; 32% (66/208) ^b	N/A	N/A

a – culture after incubation in Weybridge TEM; b – culture directly from PBS; c – passive filtration; N/A – not applicable

1.5.5. Biochemical Phenotyping

While there are a number of biochemical methods for defining *Campylobacter* species, published reports have slight variations in methods and media for the growth and testing of the isolate. These differences may affect the identification and diagnosis of infections. A defined set of test methodologies and result parameters is needed for consistency across laboratories.

Isolations of *Campylobacter*-like organisms are characterised according to the following biochemical tests: growth at 25°C and at 42°C, oxidase and catalase, growth in 1% glycine, H₂S production in a 0.02% cysteine medium, and resistance to nalidixic acid (Table 4.). It has however, been noted that there are strains within each species which produce anomalous results on defining tests confounding the identification of isolated bacteria. Standard test inoculums and media need to be used for biochemical differentiation to reduce discrepant results, as higher concentrations (10⁷ – 10⁸ CFU/ml) have been shown to produce false positives on the *Campylobacter* strains tested (On and Holmes 1991).

Table 4. Recommended differentiating tests for commonly isolated *Campylobacter* species from bovine preputial samplings (Modified from ((Vandamme 2005); Veron and Chatelain, 1973))

Species	Oxidase	Catalase	25°C	42°C	TSI H ₂ S	H ₂ S broth ^a	Glycine	Nalidixic Acid	Cephalothin
<i>C. fetus</i> subsp. <i>fetus</i>	+	+	+	- ^b	-	+	+	R	S
<i>C. fetus</i> subsp. <i>venerealis</i> biovar <i>Venerealis</i>	+	+	+ ^c	-	- ^d	-	-	R ^e	S
<i>C. fetus</i> subsp. <i>venerealis</i> biovar <i>Intermedius</i>		+	+	-	-	+		R	S
<i>C. jejuni</i>	+	V	-	V	-	+	V	S	R
<i>C. hyointestinalis</i>	+	+	-	+	V	+	V	R	S
<i>C. sputorum</i>	+	V	-	+	+	+	+	V	S
<i>Arcobacter cryaerophilus</i> ^f	+	+	+ ^g	- ^g	-	-	-	S	V

TSI – triple sugar iron slope; a – H₂S production in a 0.02% cysteine broth using lead acetate paper; b – thermo-tolerant *C. fetus* strains identified; c – strains that do not grow at 25°C have been identified; d – typically negative, though strains have been shown to produce H₂S in the presence of cysteine; e – typically resistant, though variable antibiotic results have been identified; f – as per (Schroeder-Tucker, Wesley et al. 1996); g – temperature sensitivity under aerobic conditions.

1.5.5.1. Temperature Sensitivities

Temperature variance is a differentiating biochemical test with *C. fetus* subsp. *venerealis* growth predominantly positive at 25°C and negative at 42°C (OIE 2012). Enteric *Campylobacters* such as *C. jejuni* and *C. coli* are able to grow at 42°C but not at 25°C while venereal bacteria appear to require a lower ambient temperature (Smibert and von Graevenenitz 1980). The possibility of contamination in samples from thermophilic *Campylobacter* species has not been addressed in the literature. Contending detection of *C. sputorum* in penile samples which are thermophilic, may require differential incubation in order to favour growth of *C. fetus* subsp. *venerealis* (Indjein 2013). While *C. fetus* does not typically grow at 42°C, thermo-tolerant strains have been isolated from humans (Klein, Vergeront et al. 1986) as well as cattle (Schulze, Bagon et al. 2006). Further, identification of *Arcobacter* species amongst strains collected from bull penile sheaths that were originally identified as *C. fetus* subsp. *venerealis* highlights the need to extend differentiating characteristics (Kiehlbauch, Brenner et al. 1991, Indjein 2013). Aerotolerance is not suggested as a differentiating test in OIE guidelines, however, *Arcobacter* species that are able to grow microaerobically can otherwise be confused with *C. fetus* subsp. *venerealis* strains due to similar biochemical characteristics. *Arcobacter cryaerophilus* have been shown to be tolerant of 25°C and 37°C microaerobically, but will not grow at 42°C under either atmosphere (Schroeder-Tucker, Wesley et al. 1996).

1.5.5.2. Glycine Tolerance

Identification of the correct *Campylobacter* subspecies is necessary due to the differences in epidemiological profiles of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. It has however been argued that the genetic variation based on DNA hybridization studies between the two subspecies is insufficient to create a distinct new taxonomy (Harvey and Greenwood 1983). While the biochemical characteristics of the two subspecies are almost indistinguishable except for growth in the presence of 1% glycine, the study questioned whether this was a significant enough differentiation given the identification of *C. fetus* subsp. *venerealis* strains shown to be glycine tolerant. Subsequent research using pulse-field gel electrophoresis (PFGE) showed that *C. fetus* subspecies had distinguishing genomic sizes (Salama, Garcia et al. 1992). *C. fetus* subsp. *fetus* strains (n=7) had a genome size of 1.1 MB and *C. fetus* subsp. *venerealis* (n=7) 1.3 MB. *C. fetus* subsp. *venerealis* strains were further classified into those that could tolerate up to 1% glycine (n=5), in this study identified as *C. fetus* subsp. *venerealis* biovar Intermedius which were 1.5 MB. While these strains could be those acquiring phages for glycine tolerance (Chang and Ogg 1971), typically *C. fetus* subsp. *venerealis* and its biovars have been shown to be glycine negative (Veron and Chatelain 1973, On 1996).

1.5.5.3. Hydrogen Sulphide Production

While *C. fetus* is typically negative for H₂S production on a triple sugar iron (TSI) slope, testing using lead acetate paper in a 0.02% cysteine broth has been identified as a more sensitive method (Veron and Chatelain 1973). By this method, *C. fetus* subsp. *fetus* is typically positive, while *C. fetus* subsp. *venerealis* biovar Venerealis are negative. However, biovar Intermedius has been shown to be positive, which can complicate diagnosis in the light of glycine tolerant *C. fetus* subsp. *venerealis* strains which could be mistaken for *C. fetus* subsp. *fetus* (Figure 2).

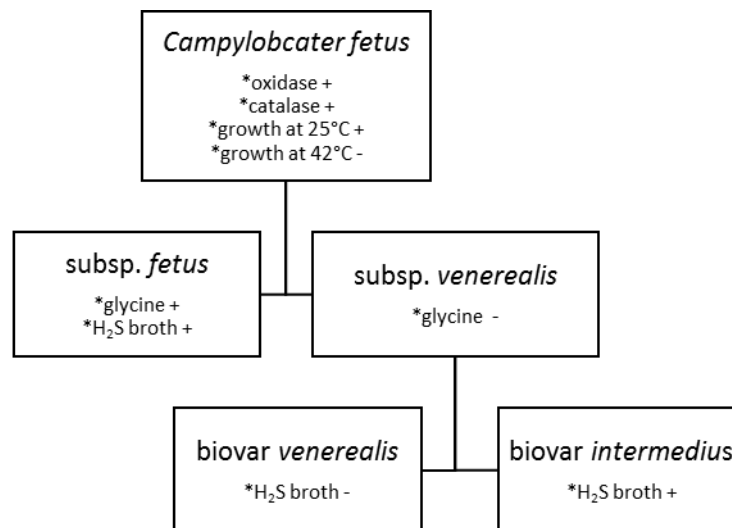


Figure 2. Schematic of biochemical differentiation of *Campylobacter fetus* subspecies and biovars showing typically implemented phenotypic characterisation for speciation at the Biosecurity Queensland veterinary diagnostic laboratory (Brisbane, Australia).

1.5.6. API Campy Kits

An API (Analytical Profile Index) test is a standardised identification system which has been optimised for use in medical microbiology laboratories for the classification of clinically relevant bacterial species. The test consists of 20 dehydrated substrates made up of 11 enzyme based tests, 6 assimilation assays and 3 susceptibility assays on two strips. The strips are inoculated with a bacterial suspension and incubated for 24 - 48 h at 37°C with the substrates able to produce reactions in the presence of bacterial metabolism or growth. The enzymatic tests require the addition of a reagent after incubation to produce the required reaction, according to the manufacturer's instructions. The reading of the assimilation and susceptibility assays is dependent on the positive growth of the succinate test (re-incubation for a further 24 h is often required for more fastidious organisms such as *Campylobacter*). The semi-automated kit requires the input of the reactions which are read according to a standard reading table to produce a binary identity. This is used to identify the organism through an online program which produces the strain identification result.

Although API Campy kits have not been used for the diagnosis or differentiation of *C. fetus* subsp. *venerealis*, it has been used successfully in identifying *C. jejuni* with a 100% correlation between API results and independent biochemical tests (Huysmans, Turnidge et al. 1995). However, the kit was unable to correctly identify 26% (5/19) of *C. coli* and 33% (1/3) of *C. lari* strains which may translate to other *Campylobacter* species. In a similar study, testing the same species there was a

75% correlation between the independent assays and API test for *C. coli* and 0% of the *C. lari* strains (Reina, Ros et al. 1995). As noted by the authors, this test has the potential to provide easier, less subjective identification of *Campylobacter* strains. However, the mislabelling seen with the API test in these studies necessitates the use of independent tests.

The kits are a low cost, readily available identification apparatus already utilised by most diagnostic labs for other more common bacteria, and could provide another analytical tool if further research proved it effective. The consistency achievable through the use of such a standardized assay system such as API kits as opposed to independent tests may reduce variation between veterinary laboratories testing bacterial isolations under different conditions.

1.5.7. Biolog Phenotypic MicroArray

Biolog Phenotype Microarray (PM) is a physiological test used to ‘define’ bacterial characteristics through 1,920 unique assays including carbon, nitrogen and biosynthetic pathways; ion, osmolarity and pH and chemical sensitivity assays. The combination of results produces a comprehensive profile for distinction of different bacterial species and strains (Bochner 2009).

The assays are performed in 96-well plates containing alternative sources of the particular assay product in each well, which, if catabolised by the inoculated bacteria, produces reduced nicotinamide adenine dinucleotide (NADH) and a colour change graph to demonstrate the resulting phenotype. The test is unique in that it measures respiration levels and not growth, to give a more accurate representation of the effect of the specific culture condition and allowing analysis of the organism’s metabolic processes and cellular pathways (Bochner 2009).

PMs have been used extensively to produce global phenotyping of bacterial species functions. Researchers have used the tool to define *Escherichia coli* regulatory systems (Zhou, Lei et al. 2003), to detect phenotypic changes in carbon metabolism in mutant *E. coli* strains (Funchain, Yeung et al. 2000), to determine the loss or gain of metabolic functions in *Salmonella* stored for extended periods of time (Tracy, Edwards et al. 2002) and to determine metabolic changes following gene knockout studies in different microbes (Perkins and Nicholson 2008).

Previous studies on *Campylobacter* species have researched the effect of temperature on carbon utilization in *C. jejuni* and *C. coli* (Line, Hiatt et al. 2010, Line, Seal et al. 2011). The research aimed to define any phenotypic changes that occurred when either bacteria passed from its host,

usually poultry or swine where the core temperature is 42°C to the human host with a core temperature of 37°C. It was hypothesised that the change in temperature might account for the colonization and activation of virulence factors. The results showed a variation in utilization of different carbon pathways at the different temperatures, but further research is needed to define the role of these differences in the bacterial virulence (Line, Hiett et al. 2010).

There is currently no published research on the use of PMs on *C. fetus*, therefore further investigation of this technique for *C. fetus* subsp. *venerealis* could provide assays for differentiation between *Campylobacter* species and strains.

1.5.8. Immunological identification

1.5.8.1. ELISA

ELISA (Enzyme-Linked Immunosorbent Assay) is a diagnostic tool available for the analysis of immune response in vaginal mucus samples (Hum, Stephens et al. 1991). The ELISA assay works by detecting IgA antibodies present in vaginal mucus samples in response to BGC infection. Antibodies from vaccination do not produce false-positive reactions as it elicits an IgM and IgG response instead. Heifers and cows have been shown, through previous ELISA testing, to be able to spontaneously clear infections over a period of a few months (McMillen, Fordyce et al. 2006). However, as ELISA screens for specific immunoglobulin antibodies for the bacteria which can remain present for up to ten months after infection, it can provide a retrospective indication of infection in a herd (Hum, Stephens et al. 1991). ELISA testing is unable to differentiate between *C. fetus* subspecies infection, but is often used as a screening tool with subsequent culture testing on any positive herds. The ELISA is unable to be used for the screening of bulls due to the lack of the required antibodies in preputial samplings (Winter 1982).

Previous ELISA testing conducted on 241 herds throughout New South Wales found the assay to be 98.5% specific in diagnosing the incidence of BGC in 34.8% of properties tested (Hum, Quinn et al. 1994). However, the study did not use a secondary diagnostic test to compare the findings. The study suggested the test only be used in herds with already reduced calving rates as a herd indicator, rather than for individual diagnosis. A second study in New Zealand found that the ELISA test could have a possible cross reaction with other *Campylobacter* species. Fifty-four bulls from 9 different herds which had heifers with high ELISA positives were tested using culture and PCR. None of the bulls were positive for *C. fetus* subsp. *venerealis*; however, four other *Campylobacter*

species were cultured from the samples. The study concluded that ELISA testing for diagnostic purposes was not recommended due to a possible non-specific immunological response caused by other *Campylobacter* species (McFadden, Heuer et al. 2004).

1.5.8.2. Agglutination Tests

Vaginal mucus agglutination tests have been previously used for the diagnosis of BGC in infected heifers as a herd test (Plastringe, Easterbrooks et al. 1953, Kendrick 1967). These tests detect levels of IgA, which can persist for extended periods after infection, making them valuable screening tools. However, cross reaction with non-viable *Campylobacter* cells used in vaccination can affect its specificity (Hazeleger, Beumer et al. 1992) reducing its reliability as a diagnostic test.

Commercial latex agglutination tests have been used for the detection of a number of *Campylobacter* species. These kits use immunoglobulins against different *Campylobacter* species coated on latex particles. Available researched kits include Campyslide (BBL Microbiology Systems, Cockeysville, MD), Microgen *Campylobacter* M46 (Microgen Bioproducts, Surrey, United Kingdom), Microscreen (Mercia Diagnostics, Shalford, United Kingdom), Meritec-Campy (also known as PanBio-Campy) (Meridian Diagnostics, Cincinnati, OH) and Dryspot *Campylobacter* (Oxoid, Basingstoke, Hampshire, England) (Hodinka and Gilligan 1988, Hazeleger, Beumer et al. 1992, Miller, Speegle et al. 2008).

The validity of these tests have been researched with studies showing that while all *Campylobacter* species tested could be correctly identified using the Campyslide system, a number of false positives with *P. aeruginosa* were seen (Hodinka and Gilligan 1988). A second study found that the Campyslide system produced the lowest number of positives from chicken product samples, with only 15% of positives detected, while the Meritec and Microscreen systems were able to detect 63% and 69% of positive samples respectively (Hazeleger, Beumer et al. 1992). Comparison of the Microgen, Dryspot-*Campylobacter* and Meritec systems on 125 different *Campylobacter* strains of seven species found 98.4%, 84.0% and 76.8% accuracy respectively (Miller, Speegle et al. 2008). Microscreen, Microgen and Dryspot *Campylobacter* are able to detect *C. fetus* immunoglobulins, but as this is not a specific differential test, its use diagnostically is not recommended (Fang, Wang et al. 1995).

1.5.8.3. Immunofluorescence

Fluorescence antibody testing (FAT) was developed as a detection method for *C. fetus* in vaginal mucus, aborted foetal tissue and preputial samples. It was used to detect *C. fetus* subsp. *venerealis* in 61 samples from five known positive bulls and 188 samples from bulls of unknown status (Lander 1990). The detection level was 96.7% in the first group and 2.7% in the second group. Sensitivity of the FAT test appeared to be equal or greater than that of culture, which was 90.2% and 2.7% in each group respectively.

An Immunofluorescence antibody test (IFAT) was used to determine the status of vaccinated and unvaccinated animals in response to two commercial vaccines (Cobo, Cipolla et al. 2003). The study found a high level of correlation between IFAT and culture results with a few anomalous positive IFAT, culture negative results. While the authors believed IFAT was more suitable than the FAT due to increased sensitivity and decreased cost and testing time, its inability to differentiate between past or current infections or between infections caused by either subspecies, it is not recommended as a diagnostic test (OIE 2012).

1.5.9. Molecular identification

1.5.9.1. Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a genetic technique involving enzymatic digestion of chromosomes, followed by fluorescence-labelled primer amplification, and separation of the product using polyacrylamide gels to produce bands typically 50 to 500 nucleotides in length (Wassenaar and Newell 2000).

The technique has been used for the differentiation of *C. fetus* to the subspecies level in different studies (Duim, Vandamme et al. 2001, van Bergen, Simons et al. 2005), however, one study found that it was unable to distinguish between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (On and Harrington 2000). It has been shown to be highly successful in differentiation of species (and certain subspecies) within taxonomic and epidemiologically related instances, however, the labour intensive complexity of the technique and requirement of expensive software and equipment and pure cultures for analysis, makes it inadequate for most diagnostic laboratories (Wassenaar and Newell 2000, OIE 2012).

1.5.9.2. Multi Locus Sequence Typing (MLST)

Multi Locus Sequence Typing (MLST) uses nucleotide sequencing of internal housekeeping genes to characterise different bacterial species. Different sequences within the housekeeping genes are allotted as distinct alleles, which at the loci, define the sequence type (ST) (van Bergen, Dingle et al. 2005). An MLST profile for *C. fetus* was developed using 140 previously AFLP-typed isolates. The profiling identified 14 ST of which ST-4 was identified in *C. fetus* subsp. *venerealis* only (van Bergen, Dingle et al. 2005). MLST has also been used as a confirmatory test in the evaluation of real-time PCR assays with great success (Chaban, Chu et al. 2012, van der Graaf-van Bloois, van Bergen et al. 2013). Due to the robustness of the housekeeping genes, it is a reliable and accurate test for identification and subspeciation of *C. fetus* (van Bergen, Dingle et al. 2005). However, as with AFLP, due to the complexity and cost, it is not a feasible diagnostic test at this time.

1.5.9.3. Pulse Field Gel Electrophoresis (PFGE)

Pulse Field Gel Electrophoresis (PFGE) is the digestion of bacterial chromosomal DNA in agarose, to reduce random breaks, using restriction enzymes. The product is separated by gel electrophoresis to produce a genotypic profile (Wassenaar and Newell 2000). The methodology has been used for a variety of *Campylobacter* species including *C. jejuni*, *C. coli* and *C. fetus* (Salama, Garcia et al. 1992, Hum, Quinn et al. 1997, On and Harrington 2001). However, variations in electrophoresis conditions as well as restriction enzymes used across different laboratories has resulted in different PFGE profiles for the same isolates (Salama, Garcia et al. 1992, Hum, Quinn et al. 1997, Wassenaar and Newell 2000, On and Harrington 2001, Vargas, Costa et al. 2003).

PFGE was used instrumentally in the identification of *C. fetus* subspecies and biovar sizes (Salama, Garcia et al. 1992). This study showed through PFGE, that *C. fetus* subsp. *fetus* possesses a genome size of 1.1 MB, while *C. fetus* subsp. *venerealis* had a genome size of 1.3 MB and its biovar Intermedius a genome size of 1.5 MB. These results were consistent with glycine tolerance differentiation (Salama, Garcia et al. 1992). While this study lead to the belief that PFGE could be used as a distinguishing test for *C. fetus* subspecies, it was found to be inconsistent in its identification (On and Harrington 2001).

1.5.9.4. Polymerase Chain Reaction (PCR)

Difficulties in culture and phenotyping have led to the research of molecular tools that may have better sensitivity in diagnosing BGC. Polymerase Chain Reaction (PCR) assays have been widely used by diagnostic laboratories for a variety of diseases and was therefore an ideal molecular tool over AFLP or PFGE. A duplex PCR was developed with a set of primers (MG3F and MG4R) identifying *C. fetus* strains (960 bp amplicon) which was later identified as 764 bp (Muller, Hotzel et al. 2003), and a second primer set (VenSF and VenSR) specific for *C. fetus* subsp. *venerealis* (142 bp) (Hum, Quinn et al. 1997) in a duplex format for PCR. The PCR was compared with PFGE and biochemical characterisation of 99 *C. fetus* strains, biochemically phenotyped as *C. fetus* subsp. *venerealis* (n=59) and *C. fetus* subsp. *fetus* (n=40). Phenotyping, PCR and PFGE were shown to have an 80.8% correlation, with most discrepant results due to inadequate biochemical differentiation (Hum, Quinn et al. 1997).

PCR profiling of 69 *C. fetus* strains from three geographical regions were typed using the Hum PCR (Wagenaar, van Bergen et al. 2001). Biochemically, 47 *C. fetus* subsp. *fetus* and 22 *C. fetus* subsp. *venerealis* strains were identified. All strains produced the *C. fetus* specific band (960 bp) while only 15 produced the *C. fetus* subsp. *venerealis* band. The 7 strains identified as *C. fetus* subsp. *fetus* molecularly were negative on the phenotypically differentiating test growth in 1% glycine. AFLP was used to confirm the identities, supporting the PCR results. The authors noted that the misidentified strains all came from a subset of South African strains, which may indicate some evolutionary differences in geographically different strains. Conversely, one strain that was molecularly identified as *C. fetus* subsp. *venerealis* was glycine tolerant and subsequent AFLP and immunofluorescent testing was unable to resolve its identity (Wagenaar, van Bergen et al. 2001).

Similarly, confirmation of biochemical phenotyping of *C. fetus* strains in Brazil identified 29 *C. fetus* subsp. *venerealis* strains and 4 *C. fetus* subsp. *fetus* strains. Molecular identification using the Hum PCR determined that three strains had been incorrectly identified as *C. fetus* subsp. *fetus* although confirmation using PFGE could not be achieved. The authors noted that the duplex PCR was easily implemented, convenient and reliable within their laboratory (Vargas, Costa et al. 2003).

Adaptation of the Hum duplex PCR to include a *C. fetus* subsp. *fetus* specific amplicon (435 bp) was conducted in the United Kingdom (Willoughby, Nettleton et al. 2005). The PCR was used to identify 92 *Campylobacter* strains, including 76 *C. fetus* strains isolated in the UK. This study only produced a 16% and 24% correlation with phenotypic testing of *C. fetus* subsp. *venerealis* and *C.*

fetus subsp. *fetus* field strains respectively. The authors noted a possible geographic differentiation in strains in the UK that was not consistent with the molecular profile used in the developed PCR (Willoughby, Nettleton et al. 2005).

A recent review of the molecular tools available in comparison using an ISC*fe* primer set showed that sensitivity of commonly used PCR assays ranged from 53% - 97%, and specificity from 83% - 100% (van der Graaf-van Bloois, van Bergen et al. 2013). Interestingly, the most commonly used assay – Hum PCR, was only able to detect 58% (35/60) *C. fetus* subsp. *venerealis* strains and had the lowest specificity. The highest combination of sensitivity and specificity was shown with the Abril PCR (Abril, Vilei et al. 2007), with 97% and 100%, respectively. It was the only PCR comparable with the ISC*fe* 1 and 2 primer sets, which produced 97% - 100% across the two categories. Identification of the 143 strains tested in the study were confirmed by AFLP and MLST, which while accurate, are not feasible as rapid, cost efficient diagnostic tests. The tested nahE PCR, which is only *C. fetus* specific was shown to have 100% specificity and sensitivity (van der Graaf-van Bloois, van Bergen et al. 2013). While not able to distinguish between the two subspecies, it allows confident confirmation to the species level which can still be used in diagnosis as either species present in the bull penile sheath may have the ability to produce disease.

However, as inconsistencies in PCR results have been shown, it is prudent to supplement diagnosis with microbiological results. This requirement can be exemplified by an unusual case from New Zealand (Spence, Humphrey et al. 2011). A bull with positive *C. fetus* subsp. *venerealis* culture and PCR from faecal samples was negative on preputial samples. Further testing showed that while positive on an initial real-time PCR (McMillen, Fordyce et al. 2006) the biochemical characterisation was inconclusive. The isolates were then profiled by the OIE Reference Laboratory, where an initial duplex PCR (Hum, Quinn et al. 1997) carried out was *C. fetus* subsp. *venerealis* positive, but a second PCR test (Abril, Vilei et al. 2007) was negative. Further profiling using 16S rRNA and AFLP sequencing followed by specific PCR analysis for confirmation, concluded that the identity of the sample was in fact *C. hyointestinalis*. Diagnosis of a bull with this species is of little concern compared with the implications of diagnosing a bull with a *C. fetus* subsp. *venerealis* infection. The author has acknowledged that the lack of PCR validity is of great concern, with possible unnecessary restrictions placed on farmers who otherwise have infection free herds (Spence, Humphrey et al. 2011). The cross-reaction shown between the *C. hyointestinalis* samples with *C. fetus* and subspecies specific assays shows that molecular identification needs to be

supplemented with microbiological phenotyping and cannot be used as an independent diagnostic test (Spence, Humphrey et al. 2011).

The use of boiled lysates in diagnostic real time PCR has been shown to be unreliable (Indjein 2013, Sanhueza, Heuer et al. 2014). No correlation was found between PCR results and pregnancy rates of herds in New Zealand when testing 222 bulls using the McMillen PCR (Sanhueza, Heuer et al. 2014). Molecular results in these instances emphasize the need for gDNA to be used in PCR testing which requires optimised culture techniques to allow this to happen. With potential inadequacies with biochemical differentiation, molecular testing could be better used complementarily to culture as a differentiation technique rather than screening of boiled lysates.

1.5.9.5. Plasmids and Virulence Genes

Genes associated with virulence characteristics have been identified in different *Campylobacter* species, with little information on *C. fetus* strains, including adhesins, cytotoxins, flagellin genes, type IV secretion systems, and surface layer proteins. Each of these play a role in the associated virulence of a particular strain in mediating bacterial cell attachment and invasion of the host (Wassenaar and Newell 2000).

Adhesins are proteins associated with the adherence to the host, as well as release of toxin secretions. Research into different *Campylobacter* species have identified a range of proteins associated with adhesion production, however those found in *C. fetus* are unique implying a different adhesion mechanism comparatively, which may explain its association with the reproductive tract (Moolhuijzen, Lew-Tabor et al. 2009).

Many bacterial species including *C. jejuni* and *C. coli* have been show to possess cytotoxins, which destroy targeted cells within the host causing associated pathogenic symptoms. While sequences encoding for toxins have been identified in the *C. fetus* subsp. *venerealis* genome, there has been no identification of its association with any resulting pathogenesis (Wassenaar 1997, Moolhuijzen, Lew-Tabor et al. 2009).

Flagellin genes associated with motility and host cell invasion, *flaA* and *flaB* have been found in *C. coli*, *C. jejuni* and *C. fetus* subsp. *fetus* indicating a level of conservation across the genus. One gene (*flaB*) has been identified in *C. fetus* subsp. *venerealis* biovar Intermedius but not biovar Venerealis

(Moolhuijzen, Lew-Tabor et al. 2009). *Fla* genotype modifications have been observed under the acquisition of antibiotic resistance or other mechanisms making it unsuitable for use as genetic marker (Wassenaar and Newell 2000).

Type IV membrane-associated secretion systems are used by both Gram positive and negative bacteria for targeted virulence factor delivery (Kienesberger, Trummer et al. 2011). The genomic island on which this secretion system is situated, also holds mobility genes which may assist with gene movements between plasmids and the core genome (Gorkiewicz, Kienesberger et al. 2010). Tetracycline and streptomycin resistance genes have been found in *C. fetus* subsp. *fetus* strains, also located on a pathogenicity island thought to be associated with mobility (Abril, Brodard et al. 2010).

Surface layer proteins (SLP), associated with host cell immune response evasion, have been identified in *C. fetus*. These have been shown to be associated with bacterial virulence by reducing cell lysis and phagocytosis (Blaser, Smith et al. 1988, Thompson 2002). Animal studies where rats and sheep inoculated with *C. fetus* subsp. *fetus* strains either possessing or missing the SLPs have shown higher recovery rates and abortive incidences relating to strains possessing the SLP (Pei and Blaser 1990, Grogono-Thomas, Dworkin et al. 2000).

1.6 Preservation Techniques

Initial studies into the preservation of *Campylobacter* species was based on freeze-drying (Stockton and Newman 1950, Jakovljevic 1972, Mills and Gherna 1988). The effectiveness of this method was studied by testing the storage of seven *C. fetus* subsp. *fetus* strains, one *C. fetus* subsp. *venerealis* strain and one *C. jejuni* strain (Mills and Gherna 1988). Storage at -20°C, -65°C and in liquid nitrogen was compared with the first two being stored in both 10% glycerol and brucella albimi broth (a previous commonly used broth for the culture of fastidious organisms such as *Brucella* spp.). The effects of freeze-drying using rapid or slow cooling were also tested with different cryopreservation media. Viability was lowest at -20°C, lasting one month in brucella albimi and seven months in glycerol. The next least effective was storage at -65°C, where there was no difference in effects of storage medium. Freeze-drying proved more effective than storage at -20°C and -65°C, with slow cooling of cultures providing greater recovery than rapidly cooled cultures and remained viable up to two years later. The study clearly showed that by comparison, storage in liquid nitrogen was the most effective by allowing cultures to remain viable after freezing for up to four years after being stored in brucella albimi growth media (Mills and Gherna 1988).

An evaluation of five different storage methods for *C. jejuni* did not include freeze-drying or liquid nitrogen. The authors noted that due to the fact that not all laboratories have access to the equipment necessary for those storage methods, more easily accessible methods would be trialled (Gorman and Adley 2004). Therefore more commonly used preservation methods were assessed, including CryoBank™ storage beads with cryopreservation solution or lysed horse blood, FBP (ferrous sulphate, sodium bisulphite, sodium pyruvate) medium (George, Hoffman et al. 1978) and 15% glycerol vs. 50% glycerol on five different isolates. Resuscitation from storage was checked at 1, 2, 3, 6, 9 and 12 months. The study showed that FBP medium was the most effective over the 12 month period, with 100% recovery of isolates from a temperature of -85°C, while there was 80% recovery at -20°C (Gorman and Adley 2004).

Due to the difficulty in isolating this bacteria and ensuring correct identifications, appropriate storage methods must be utilized to allow continued access to samples. It is also important to ensure that when samples are stored, purity checks are conducted, as well as storing an adequately high concentration of cells to allow optimal recovery. Samples can often become non-culturable after prolonged periods at -20°C or -85°C and with difficulties in isolating the samples to begin with, it is necessary to maintain samples that have been isolated, to ensure availability for further profiling as new tests are developed (Tholozan, Cappelletti et al. 1999, Gorman and Adley 2004).

1.7 Animal Models

Animal models have been used throughout science for centuries, to determine the causes and effects of pathogens and their curatives. They provide a cheaper, quicker and usually more controlled setting than in the hosts. Previous studies on the development of an infection model for *C. jejuni* and *C. coli* in primates and domestic animals proved unsuccessful (Andress, Barnum et al. 1968, Al-Mashat and Taylor 1980). Guinea pigs, the rodent used in the study of *C. fetus* species, are used in part for its mid-length gestation (60-69 days) which allows breeding, infection and isolation to happen within a fraction of the time required to monitor infection in cattle. This along with the costs associated with large animal research including cost of animals and care, period of time required for gestational studies and ethical considerations, means that small animal models are a more favourable option. In guinea pigs specifically, its longer gestation, multiple foetuses of larger size allow easier sampling and extraction of amniotic fluids for testing as well as analysis of infection symptoms.

Guinea pigs have been used in the study of *Campylobacter* species for almost 60 years with success (Ristic and Morse 1953, Bryner 1976, SultanDosa, Bryner et al. 1983, Burrough, Sahin et al. 2011). Guinea pigs have been shown to be susceptible to teratogenic stimulants, and are sensitive to *Campylobacter* infections as well as effective vaccine responders (Coid, O'Sullivan et al. 1987, Burrough, Sahin et al. 2009). An initial study by Ristic was successful in producing a model for the study of BGC infection in pregnant guinea pigs (Ristic and Morse 1953).

In cattle, the disease symptoms including sporadic abortions and more common infertility (OIE 2012). Although infertility as symptom of disease has not been tested using the guinea pig model, abortion as a measure of strain variations and infection mechanism has been successfully studied. Likewise, the infection route may differ – venereally transmitted compared to oral or intraperitoneal inoculation, however, the model has been shown to be successful in allowing progression of infection so that the effects of the bacteria can be assessed.

While guinea pigs do not share the same reproductive structural specificities, such as possessing a single discoidal placenta (Kaufmann and Davidoff 1977) compared with the bovine cotyledonary placenta (Schlafer, Fisher et al. 2000), the placentas still perform the same functions, providing oxygen and nutrients and working as a materno-foetal attachment site within the uterus. This is of importance as based on previous studies where placental necrosis and haemorrhage after infection with *Vibrio fetus* were seen to be indicators of abortion (Ristic and Morse 1953).

1.7.1. Small Animal Models

Initially, small animal models were used as a diagnostic tool for determining the presence of '*V. fetus*' in bulls (Adler 1953, Power 1954). Semen samples, assumed to be infected, were intra-vaginally inoculated into guinea pigs in oestrus which were euthanized and cultured 5-6 days post inoculation. The method was identified as a test to be used in conjunction with heifer test-mating for suspected bulls. While this method was not routinely used due to the need for 5-6 guinea pigs per bull tested, it showed the viability of the bacteria in the guinea pig reproductive system. Further studies into '*V. fetus*' aimed to elucidate abortion mechanisms and identify infection mechanisms as shown in Table 5.

The initial study (Ristic and Morse 1953) showed that a pregnant guinea pig model could be used to successfully study the abortion mechanism of *Campylobacter* species. The study compared the pathogenesis of combined '*V. fetus*' strains from bovine and ovine sources via four different routes; intraperitoneal, oral, subcutaneous and intra-vaginal. It also aimed to determine the guinea pig response to a combination of '*V. fetus*' and *Brucella abortus* infection. The addition of *B. abortus* was due to a previous study where it was postulated that it provided a compromised system that was susceptible to infection by other bacterial species (Schroeder 1920). Dams were inoculated on multiple days at approximately 4-7 weeks of gestation and were euthanized at time points from 1 – 34 days after inoculation. Concurrent non-pregnant dams were inoculated in each group. '*V. fetus*' inoculation via the intra-peritoneal route resulted in 4/7 abortions, oral inoculation 2/9 abortions, subcutaneous injection 2/7 abortions and intravaginal inoculation 1/6 abortions. Inoculation of dams with *B. abortus* only resulted in one abortion (n=6 dams), while dams inoculated with both *B. abortus* and '*V. fetus*' produced abortions in 5/6 dams. In these animals, '*V. fetus*' was isolated from all dams, with *B. abortus* only isolated from two. All non-pregnant dams were culture negative at the endpoint, except two animals inoculated with both *B. abortus* and '*V. fetus*'. The study concluded that the difference in abortion rates between the '*V. fetus*' infection alone compared to *B. abortus* and '*V. fetus*' was not significant enough to support Schroeder's theory, and that while each inoculation route was able to produce abortions, the intraperitoneal route was most effective. Some of the pathological findings in the study included oedema and necrosis of the uterus, placenta and foetuses, cysts at the foetal-placental attachment site, haemorrhage within the endometrial tissue and inflammatory reactions, though not specified of what type (Ristic and Morse 1953).

Subsequent studies defined variations in *Campylobacter* strains based on percentage abortions as well as re-isolation from internal organs (SultanDosa, Bryner et al. 1983). The model assessed

Campylobacter strains from bovine, ovine, swine, avian and human sources and was successful in identifying pathogenic strains. Pregnant guinea pigs at week 3-4 of gestation were inoculated with five bovine *Campylobacter* strains, amongst a host of other *Campylobacter* species strains. The inoculum for each strain was prepared at a high (1.4×10^8 CFU/ml), medium (1.4×10^4 CFU/ml) and low concentration (1.4×10 CFU/ml) in Brucella broth. Guinea pigs were monitored twice daily for 21 days or until abortion occurred. The guinea pigs were then sampled under anaesthesia and euthanized. Isolation from blood, gall bladder, spleen, kidney, liver, uterus horns and placentas were assessed by culture. The average time to abortion from inoculation was 7 days with the latest abortion at 17 days. It was found that ovine *C. fetus* subsp. *fetus* isolates appeared to be more pathogenic than bovine *C. fetus* subsp. *venerealis* isolates, with avian *C. coli* isolates shown to be least pathogenic. The abortion rates were dose dependent, with the highest concentration eliciting the quickest and most number of abortions. The study found that the most prevalent incidences of bacterial colonization were in the uterus, with all except the two avian isolates being re-cultured from the area. The study concluded, based on culture results, that the bacteria caused a 'transient bacteraemia' which allowed early isolations from the blood and gall bladder (SultanDosa, Bryner et al. 1983).

This model was used in a study to test *Campylobacter* isolates from man (*C. fetus* subsp. *fetus* and *C. jejuni*). The study found that while virulence variation was shown in the pregnant guinea pig model, the survival of the isolates *in vitro* in human and guinea pig serum differed. This suggested that while the model was useful for the pathological study of the bacteria, its correlation to human disease may not be as accurate (Coid, O'Sullivan et al. 1987).

The pregnant guinea pig model was then used to test the abortifacient roles of *C. jejuni* and *C. coli* (Taylor and Bryner 1984). The study found up to an 87% abortion rate with the former and up to a 53% abortion rate with the latter. The effectiveness of vaccination against *C. fetus* strains was then determined by measuring the correlation between antigen dose, strain and adjuvant type (Bryner, Firehammer et al. 1988). This study tested the combination of 20 differing monovalent vaccines in Freund complete adjuvant, Freund incomplete adjuvant and aluminium hydroxide gel adjuvant. It showed that protection was proportional to dose, while a mineral oil adjuvant produced higher antibody titres than an aluminium-hydroxide adjuvant.

Most recently, the developed model has been used to effectively determine the pathogenicity of *C. jejuni* strains (Burrough, Sahin et al. 2009) and subsequently the effectiveness of two different commercial vaccines (Burrough, Sahin et al. 2011) against *C. jejuni*. The first study used intra-

peritoneal inoculation of 17 guinea pigs (5 controls) and oral inoculation of 41 guinea pigs (5 controls) at week 3-4 of gestation followed by euthanasia at day 21 post-inoculation. Intra-peritoneal inoculation once again proved more effective with 10/12 inoculated dams aborting and only 6/36 orally inoculated dams aborting (Burrough, Sahin et al. 2009). The second study aimed to test the reaction of vaccinated dams to inoculation within 48 h. Fifty-one guinea pigs were vaccinated with one of two different commercial vaccines twice, two weeks apart or sham injected. Inoculation with 1.0×10^6 CFU/ml *C. jejuni* was administered at 10 days post vaccination. At 48 h only one guinea pig from the sham injected group produced abortions, and all animals were euthanized. The causative bacterium was isolated in at least one sample (placenta, blood, uterus, liver or bile) in 26/51 guinea pigs (Burrough, Sahin et al. 2011).

The validity of the guinea pig model was assessed through the abortion rate, the ability to re-culture the organism and the visualization of the organism within the tissues. Abortion rates differed per *Campylobacter* species, as was expected, and depending on the inoculation route and dose (Ristic and Morse 1953, SultanDosa, Bryner et al. 1983). Observation of the bacteria within the tissues has used Warthin-Starry silver staining (Warthin and Starry 1922) to observe '*C. pylori*' (Bayerdorffer, Oertel et al. 1989) and *C. fetus* (Fox, Murphy et al. 1982) but has not been used for *C. fetus* subsp. *venerealis*. Immunohistochemical staining for *C. fetus* and *C. jejuni* have been used successfully to visualize bacteria within slides (Campero, Anderson et al. 2005, Anderson 2007, Burrough, Sahin et al. 2009, Morrell, Barbeito et al. 2011). One study found a 100% correlation between culture results and immunohistochemical visualisation of *C. fetus* with no false negatives in control foetal slides (liver, heart, spleen, lung, intestine, muscle etc.) (Morrell, Barbeito et al. 2011). The lectin-histochemical staining concentrated on two samples of foetal lungs and involved the use of a peanut agglutinin with seven different lectins. The study also used PCR to validate the culture and immunohistochemistry results, and found at least one sample per foetus (lung, abomasal fluid or intestine) to be PCR positive. The correlation of culture with immunohistochemistry and PCR in this study implies a possible complimentary diagnostic use of these tests for conclusively identifying BGC.

Table 5. Overview of studies using small animal models for the identification of *Campylobacter* parameters in infection characteristics.

Study	Source	Species ^A	Route	Dose	Vaccinated	Inoculum Volume	Number ^B	Gestation Age	Timeline	Abortive (%)	Recultured	Microscopy	Biochemical/ molecular ID	Histology
(Ristic and Morse 1953)	Ovine-Bovine	<i>Vibrio fetus</i> ^C	IP	McFarland 3	no	1ml x 4 alternate d	7	4-7 w	25 d	yes (57%)	yes	yes	no	yes
			IVA	McFarland 3	no	1ml x 5 alternate d	6	4-7 w	18 d	yes (17%)	no	yes	no	yes
			PO	McFarland 3	no	2ml x 6 d	9	4-7 w	11 d	yes (22%) ^F	no	yes	no	yes
	Bovine	<i>Brucella abortus</i> ^D <i>B. abortus</i> ; ' <i>V. fetus</i> ' ^E	SC	McFarland 3	no	1ml x 5 alternate d	7	4-7 w	14 d	yes (29%) ^F	yes	yes	no	yes
			SC	10 ⁸	no	1ml	7	4-7 w	34 d	yes (14%)	yes	yes	no	yes
			SC; IP	10 ⁸ ; McFarland 3	no	1ml; 1ml x 4 alternate d	6	4-7 w	14 d	yes (83%)	yes ^G	yes	no	yes
(Ristic, Morse et al. 1954)	Ovine	<i>Vibrio fetus</i>	IVA	N/A	no	1ml x 5 alternate d	10	N/A ^H	3 m	yes (20%)	yes	yes	no	yes
(Morse and Ristic 1954)	Bovine (aborted fetus)	<i>Vibrio fetus</i>	IP	N/A	no	1ml x 2 alternate d	45	4-8 w	16 d	yes (76%)	yes	yes	no	yes
	Ovine (aborted fetus)	<i>Vibrio fetus</i>	IP	N/A	no	1ml x 2 alternate d	5	4-8 w	16 d	yes (100%)	yes	yes	no	yes
	Bovine (semen)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	25	4-8 w	16 d	yes (40%)	yes	yes	no	yes
	Bovine (vaginal mucus)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	20	4-8 w	16 d	yes (50%)	yes	yes	no	yes
	Bovine (preputial wash)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	5	4-8 w	16 d	yes (60%)	yes	yes	no	yes
	Bovine (aborted fetus)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	10	4-8 w	16 d	yes (60%)	yes	yes	no	yes
	Ovine (aborted fetus)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	30	4-8 w	16 d	yes (63%)	yes	yes	no	yes
	Porcine (dysentery)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	5	4-8 w	16 d	yes (100%)	yes	yes	no	yes
	Human (blood)	<i>Vibrio fetus</i>	IP	N/A	no	1ml	10	4-8 w	16 d	yes (70%)	yes	yes	no	yes
(Bryner, Foley et al. 1978)	Ovine	<i>C. fetus</i> subsp. <i>jejuni</i>	IP	5 x 10 ⁹ - 5 x 10 ⁹	no	1ml	95	4-5 w	21 d	yes (0-100%) ^J	yes	no	no	no
			IP	10 ³ - 10 ⁵	yes	1ml	67	4-5 w	21 d	yes (0-80%)	yes	no	no	no
(Bryner, Foley et al. 1979)	Bovine	<i>C. fetus</i> subsp. <i>intestinalis</i>	IM	5 - 8 x 10 ⁴	yes	1ml	505	4-5 w	21 d	yes (11-100%) ^K	yes	no	no	no
			IM	5 x 10 ⁴	no	1ml	80	4-5 w	21 d	yes (100%)	yes	no	no	no
(SultanDosa, Bryner et al. 1983)	Bovine	<i>C. fetus</i> subsp. <i>venerealis</i>	IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	30	3-4 w	21 d	yes (73%)	yes	yes	no	no
	Bovine	<i>C. fetus</i> subsp. <i>fetus</i>	IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	15	3-4 w	21 d	yes (60%)	yes	yes	no	no
	Bovine		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	30	3-4 w	21 d	yes (13%)	yes	yes	no	no
	Ovine		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	30	3-4 w	21 d	yes (80%)	yes	yes	no	no
	Human		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	60	3-4 w	21 d	yes (40%)	yes	yes	no	no
	Human		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	15	3-4 w	21 d	yes (13%)	yes	yes	no	no
	Porcine		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	45	3-4 w	21 d	yes (30%)	yes	yes	no	no
	Avian		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	30	3-4 w	21 d	yes (10%) ^L	no ^o	no	no	no
(Taylor and Bryner 1984)	Ovine	<i>C. jejuni</i>	IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	30	N/A	N/A	yes (73-87%)	N/A	no	no	no
	Human		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	45	N/A	N/A	yes (20-53%)	N/A	no	no	no
	Bovine		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	30	N/A	N/A	yes (7-20%)	N/A	no	no	no
	Porcine	<i>C. coli</i>	IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	45	N/A	N/A	yes(13-53%)	N/A	no	no	no
	Human		IP	1.4 x 10 ⁻¹⁰ - 1.4 x 10 ⁻⁸	no	1ml	15	N/A	N/A	yes (13%) ^L	N/A	no	no	no

Study	Source	Species ^A	Route	Dose	Vaccinated	Inoculum Volume	Number ^B	Gestation Age	Timeline	Abortive (%)	Recultured	Microscopy	Biochemical/ molecular ID	Histology
	Avian		IP	1.4 x 10 ⁹ - 1.4 x 10 ⁸	no	1ml	30	N/A	N/A	no ^L	N/A	no	no	no
(Coid, O'Sullivan et al. 1987)	Human	<i>C. fetus</i> subsp. <i>fetus</i>	PO	10 ⁹	no	1ml	13	6-7 w	12 d	no	yes ^M	no	no	no
			IM	10 ⁹	no	1ml	42	6-7 w	12 d	yes (40%)	yes ^M	no	no	no
			IP	10 ⁵	no	1ml	12	6-7 w	12 d	yes (50%)	yes ^M	no	no	no
			IP	10 ⁹	no	1ml	8	6-7 w	12 d	no	no	no	no	no
		<i>C. jejuni</i>	PO	10 ⁹	no	1ml	12	6-7 w	12 d	no	no	no	no	no
			IM	10 ⁹	no	1ml	36	6-7 w	12 d	yes (6%)	no	no	no	no
			IP	10 ⁵	no	1ml	12	6-7 w	12 d	yes (30%)	yes ^M	no	no	no
			IP	10 ⁹	no	1ml	6	6-7 w	12 d	yes (50%)	yes ^M	no	no	no
(Bryner, Firehammer et al. 1988)	Bovine	<i>C. fetus</i> subsp. <i>venerealis</i>	IP	100 MAD ₅₀	yes ^N	1ml	741	16 - 33 d	21 d	yes	yes	no	no	no
	Bovine	<i>C. fetus</i> subsp. <i>fetus</i>	IP	100 MAD ₅₀	yes ^N	1ml	494	16 - 33 d	21 d	yes	yes	no	no	no
	Ovine	<i>C. fetus</i> subsp. <i>fetus</i>	IP	100 MAD ₅₀	yes ^N	1ml	30	16 - 33 d	21 d	yes	yes	no	no	no
(Burrough, Sahin et al. 2009)	Ovine	<i>C. jejuni</i>	IP	1.5 x 10 ⁵	no	1ml	10	3-4 w	21 d	yes (100%)	yes	no	no	yes ^O
			PO	5.5 - 6.0 x 10 ⁸	no	1ml	20	3-4 w	21 d	yes (30%)	yes	no	no	yes ^O
	Human	<i>C. jejuni</i>	PO	5.9 x 10 ⁸	no	1ml	10	3-4 w	21 d	no	yes	no	no	yes ^O
(Burrough, Sahin et al. 2011)	Ovine	<i>C. jejuni</i>	IP	7.5 x 10 ⁵ - 1.0 x 10 ⁶	yes ^P	1ml	51	N/A ^Q	48 h	N/A	yes	no	no	yes

IP - intra-peritoneal; IM - intramuscular; IVA - intravaginal; SC - subcutaneous; PO - orally; N/A - not available; h - hours; d - days; w - weeks; m - months

^A - as noted within the study, regardless of availability of supporting biochemical or molecular data; ^B - number of pregnant animals only; ^C - identification and taxonomy not able to be correlated due to nomenclature changes; ^D - USDA reference strain; ^E - concurrent *B. abortus* and '*V. fetus*' infection was tested by inoculation with *B. abortus* followed by inoculation with '*V. fetus*' 1 week later; ^F - remaining dams went to term; ^G - '*V. fetus*' reisolated from all 6; *B. abortus* reisolated in 2 animals; ^H - guinea pigs were inoculated prior to housing with males for mating; ^J - proportional correlation with infective dose to number of abortions and time to abortion; ^K - variations dependent of vaccination; 10 different types used; ^L - *Streptococcus* isolated only; ^M - results only shown for 24h bacteraemia culture with suggestions culture was carried out on all tissues at endpoint; ^N - different vaccine strains and adjuvant types tested; ^O - first study to show placental localization and distribution of *Campylobacter* spp. as well as immunohistochemistry evidence of bacterial tissue infiltration; ^P - two vaccines and 1 experimental bacterin tested; ^Q - animals were sourced from a breeding facility where they had been recently mated, ultrasound was performed prior to vaccination/ inoculation however at the endpoint 32 animals were found to be not pregnant, and 19 pregnant.

1.7.2. Large Animal Models

Testing in cattle, for the understanding of disease pathogenesis and vaccine efficacy was carried out before the use of small animal models for vaccine studies against BGC (Te Punga and Moyle 1961, Te Punga 1962, Te Punga, Boyes et al. 1964, Plastring, Kersting et al. 1966, Mitchell 1968). While it has become convention to test pathogens or vaccine responses in small animal models prior to host testing, the need for a preventative measure against the disease caused many researchers to attempt to confer protection in herds through subcutaneous injections of the causative bacteria, *C. fetus* subsp. *venerealis* (Te Punga and Moyle 1961, Te Punga 1962, Te Punga, Boyes et al. 1964, Plastring, Kersting et al. 1966, Mitchell 1968, Clark, Dufty et al. 1970). Another study found that a *C. fetus* subsp. *fetus* strain isolated from an aborted sheep foetus, was also able to produce protection in cattle against BGC, suggesting common antigens present within the two subtypes (Hoerlein and Kramer 1963).

Vaccines for the treatment of this disease are available through different manufacturers in many countries. The current vaccine available in Australia is a bivalent *C. fetus* subsp. *venerealis* biovar *Venerealis* and biovar *Intermedius* combination in a mineral oil adjuvant. The development involved the vaccination of four infected bulls with a killed *C. fetus* subsp. *venerealis* vaccine twice, five weeks apart (Clark, Dufty et al. 1977). Upon testing of the bulls two weeks after the second vaccination, they were found to be free from infection. A second study assessed protection using different *Campylobacter* strains by determining susceptibility to genital campylobacteriosis infection after vaccination with the bovine intestinal strain, *C. fetus* (Clark, Dufty et al. 1970). Twelve virgin heifers were vaccinated twice, 10 days apart and inoculated after two months with *C. fetus* subsp. *venerealis*. Heifers were then tested for positive culture after 7 days. It was found that subcutaneous injections with the intestinal *C. fetus* were able to provide protection in 11 out of 12 heifers against reproductive *Campylobacter* infection by producing a possible cross-reaction immunization. It also found that while cross-reaction antibody titres for genital infections were present, it was lower than those for the intestinal type (Clark, Dufty et al. 1970).

The concept was tested in a subsequent study where 41 bulls testing positive for *C. fetus* subsp. *venerealis* infection were vaccinated with a killed *C. fetus* subsp. *intestinalis* strain in a Freund adjuvant (Bouters, De Keyser et al. 1973). After the first injection, 30 bulls tested negative, and after the second injection all bulls were clear of infection. The study followed up with a single vaccine in the following year, and found that none of the bulls' re-established infection in that time period. Upon the recognition of the two biovars of *C. fetus* subsp. *venerealis*: *Venerealis* and

Intermedius, responsible for bovine abortions, a bivalent vaccine combining them was tested (Clark, Dufty et al. 1977). The vaccine contained equal quantities of both biovars in a mineral oil adjuvant and was found to protect young heifers and older cows from infection with either organism. The study found 100% protection against *C. fetus* subsp. *venerealis* biovar Venerealis in vaccinated animals and 92% protection against biovar Intermedius. It is still unknown which strains may produce the most effective vaccines and whether a particular species or biovar are able to confer greater protection.

1.7.3. Vaccine Development

It was discovered that subcutaneous injection of living *C. fetus* bacteria, could induce immunity in cattle (Plastring, Kersting et al. 1966, Clark 1967). This led to the development of different commercial vaccines which contained either *C. fetus* species or subspecies to be used against BGC.

In a vaccine trial carried out across Australia to determine protection levels using a *C. fetus* subsp. *fetus* biovar Venerealis vaccine against infections of biovar Venerealis and biovar Intermedius, it was found that the vaccine offered full protection against its own biovar infection, but not against biovar Intermedius infections (Clark, Dufty et al. 1976). However, a second study using a combined biovar Intermedius and biovar Venerealis vaccine against subsequent inoculation with either biovar Intermedius or Venerealis produced 92% protection in the former group and 100% protection in the latter as measured by pregnancy rates and culture (Clark, Dufty et al. 1977). Control non-vaccinated groups had 92-100% infection as measured by culture. A second study found that vaccination with biovar Intermedius only was able to confer protection in animals inoculated with 83% against biovar Venerealis and 91% against biovar Intermedius as determined by culture. Control non-vaccinated animals had an infection rate of 80% (Johns, Mater et al. 1977).

Regular assessments of vaccines are necessary to maintain product confidence and to determine efficacy levels. A study was carried out into the efficacy of two commercially available vaccines in Argentina. These vaccines contained multiple antigens of a number of bacteria and viruses including *Leptospira*, *Histophilus somni*, both *C. fetus* subspecies, bovine rinotracheitis and bovine viral diarrhoea in aluminium hydroxide adjuvant (Bioabortogen-H, Biogenesis, Argentina). Three groups of 8 heifers were vaccinated with vaccine A, vaccine B or control unvaccinated. Forty days from vaccination, the heifers were mated with an infected bull. Pregnancy rates ranged from 25-38% in vaccinated groups, with 0% pregnancy in the non-vaccinated group with all groups having similar rates of culture (Cobo, Cipolla et al. 2003). However, a second study found that vaccination

of 27 infected bulls with a *C. fetus* subsp. *venerealis* bacterin produced a 44.5% reduction in infection after just one initial injection (Foscolo, Pellegrin et al. 2005). There was a further 11.1% reduction in infected bulls after a second injection, 23 days later, bringing the total to 55.6% less infected bulls within the herd.

This showed that while this method of treatment was useful, it could not be relied on as the only mechanism, as almost half of the remaining bulls were still carriers and could continue to pass the bacteria on to heifers or other bulls. While there was an overall reduction in infection of 55.6% in this study, the decrease in infected bulls from the first vaccination to the second was quite low. The authors noted that the potential decrease in time between vaccinations may account for the low decrease between vaccinations, as a previous study of 41 infected bulls found a 70% decrease in infection after the first vaccination and a 100% decrease in infected animals after both vaccinations (Bouters, De Keyser et al. 1973). The same study suggested that animals require between 14-56 days to develop immunity after vaccination and used a 42 day interval between vaccinations compared to the 23 days used in the recent study (Foscolo, Pellegrin et al. 2005). Furthermore, another previous study noted that animals could require up to 136 days to completely clear BGC infection (Berg, Firehammer et al. 1979). A further study showed that vaccination of heifers with a dual *C. fetus* subsp. *venerealis* and *Tritrichomonas foetus* vaccine 60 days prior to mating with infected bulls was sufficient to produce between 30-70% protection against infection with *C. fetus* subsp. *venerealis* compared to 20% uninfected heifers in the control unvaccinated group (Cobo, Morsella et al. 2004). Vaccine efficacy has been shown to be dose dependent, with a study finding that 10 to 40 mg of bacteria per dose is necessary to provide immunity (Schurig, Hall et al. 1975). This was further confirmed in comparative studies where 2 to 20 mg bacterial doses found that the level of protection was directly proportional to bacterial dose (Bryner, Firehammer et al. 1988). Further studies using doses of 40 mg of bacteria was used (Clark and Dufty 1982) and most recently where a 55.6% immunity was produced in vaccinated bulls with an 18 mg dose (Foscolo, Pellegrin et al. 2005).

1.7.4. Vaccine Antigenic Effect

In the study of antigens and antigen combinations required to elicit the highest level of immunity, H-antigens (flagellar), O-antigens (heat stable) and K-antigens (heat labile) were discovered (Clark, Dufty et al. 1975, Border and Firehammer 1980). It was found that H-antigens and O-antigens were unable to produce protection in heifers, while a combination of K-antigens, usually up to five, were needed to confer protection (Border and Firehammer 1980).

A study into the concept of antigen related immunity proposed that strains may change their antigenic and pathogenic characteristics during infection leading to reduced potential efficacy of vaccines (Schurig, Hall et al. 1975). This was supported in a second study where 32 heifers infected with one of two *C. fetus* subsp. *fetus* strains and subsequently vaccinated with one of four vaccines (whole cell, water extracted, ribosomal or saline) (Schurig, Duncan et al. 1978). It was found that all vaccines were similarly effective, with infection clearing 1-6 weeks post immunization and isolates collected at week 2 of infection were all antigenically different to the inoculated strain (Schurig, Duncan et al. 1978).

Chapter 2. Aims and Significance

2.1 Summary

The failure to diagnose BGC is predominantly related to the inability to culture the causative organism from submitted samples. While studies into the identification of optimal media for the enrichment and isolation of *C. fetus* subsp. *venerealis* have been sporadically conducted, variations in specific collection parameters (including temperature, media volume) need to be assessed. The variability of transport duration, collection and transport temperatures that can be experienced in Australia differ considerably to those in Canada or the UK where previous studies on *C. fetus* subsp. *venerealis* have been conducted (Lander 1990, Lander 1990, Monke, Love et al. 2002, Chaban, Chu et al. 2012, Chaban, Guerra et al. 2013). The identification of a newer, suitable transport and enrichment media (TEM) for use in both culture and molecular testing Thomann Transport and Enrichment media (TTE) (Harwood, Thomann et al. 2009) has gone largely unnoticed in the literature with no studies comparing its efficacy with the more commonly used Lander's TEM.

The identification of a number of thermophilic *Campylobacters* as well as *Arcobacter* species isolated from preputial samples collected from an abattoir survey (Dr Indjein's PhD studies) suggests the need for differential isolation methodologies to favour *C. fetus* subsp. *venerealis* growth. While this contention between species is not apparent in the literature, due to limitations on the source and number of *C. fetus* subsp. *venerealis* strains tested in individual studies, the relevance for more specific differential isolation methodologies may be overlooked. This lack of awareness in the literature may also be due to the misdiagnosis of strains as thermo-tolerant *C. fetus* subsp. *venerealis* which, due to either deficiencies in biochemical testing methodologies or inappropriateness of characterising tests, may occur. Similarly, strains believed to be aerotolerant *Campylobacter* species may in fact be *Arcobacter* species.

These discrepancies in *C. fetus* subsp. *venerealis* identification may be resolved by expanding the biochemical testing profile as well as testing a larger subset of strains to determine the true level of robustness of each assay. This may identify tests which are more reliable for differentiation as well as potentially any new biochemical assays to incorporate in identification profiling. At the same time, the level of inherent biochemical variation in *C. fetus* subsp. *venerealis* strains can be assessed.

While the biochemical variation of *C. fetus* subsp. *venerealis* strains is important from a diagnostic view to allow detection of causative organisms of disease, determining the presence of potential virulence variation is equally important to identify strains for vaccine inclusion and efficacy studies.

Identification of this potential virulence variation could be conducted in small animal trials to reduce the burden and cost of large animal studies. Guinea pig models for *Campylobacter* species have been used for over 60 years, however, there have been few studies into *C. fetus* subsp. *venerealis* infection specifically (Ristic and Morse 1953, Morse and Ristic 1954, Ristic, Morse et al. 1954, Ristic, Wipf et al. 1954, Bryner 1976, SultanDosa, Bryner et al. 1983). Studies have also displayed variations in strains, doses, routes, timelines and assessment of infection techniques. Therefore, further investigation is required to determine the most reliable method for infection and for monitoring outcomes such as bacterial progression or strain virulence in a *C. fetus* subsp. *venerealis* model specifically.

2.2 Aims

Using microbiological methodologies only to determine the ideal transport and isolation methods for Australian diagnostic laboratories as well as a comprehensive biochemical profile for *C. fetus* subsp. *venerealis* isolates.

Identify a robust, reliable pregnant guinea pig model with defined parameters to enable the assessment of abortifacient properties of a variety of Australian *C. fetus* subsp. *venerealis* strains.

2.3 Objectives

I. Identification of optimal transport and isolation methodologies for *C. fetus* subsp. *venerealis*
Methodological approach: *in vivo* *C. fetus* subsp. *venerealis* infection trial using 28 bulls and 28 heifers was utilised to compare transport of samples in two different media (Lander's TEM and TTE). The trial location was approximately half an hour from the diagnostic lab allowing timely delivery of samples. With the trial conducted between January and May (Summer – Autumn), it provided an ideal setting to determine ambient temperature for the transport of cultures, which is an important factor for diagnostic samples received from tropical regions of Australia. State diagnostic laboratories are often located great distances from farming communities based in tropical northern areas, and samples can take days for delivery from rural areas. Isolation of *C. fetus* subsp. *venerealis* was compared with TEM subculture onto SBA (with and without a 0.45 µm filter) and *Campylobacter* selective agar. Differential incubations at 25°C and 37°C were assessed in a spiked mixed culture laboratory trial with *C. fetus* subsp. *venerealis*, a thermophilic *Campylobacter* strain (*C. jejuni*) and common contaminating bacteria such as *Pseudomonas* and *Proteus*.

II. Establishment of a comprehensive database of *C. fetus* subsp. *venerealis* biochemical characteristics

Methodological approach: All strains collected during a previous abattoir survey or diagnostically isolated during the course of this PhD research were biochemically profiled (n=152). A subset of strains of interest for inclusion in animal trials or vaccine studies were subject to antibiotic testing (n=6), API Campy kit profiling (n=42), Biolog Gen III (n=27) and Biolog PM Array profiling (n=10). These results were compared with PCR and MLST profiles, where available (completed by during Dr Indjein's PhD studies and Yusralimuna Nordin's Honours study).

III. Development of a reliable small animal model for the assessment of *C. fetus* subsp. *venerealis* strains

Methodological approach: A preliminary trial was conducted using 35 pregnant guinea testing three strains. The dose was determined by testing three different concentrations (low 10^4 CFU/ml, medium 10^7 CFU/ml, high 10^9 CFU/ml) and two different routes were assessed; intra-peritoneal or intra-vaginal inoculation. Upon the identification of the correct parameters, the model was used to identify a protective dose of an experimental vaccine against *C. fetus* subsp. *venerealis* infection using 22 animals vaccinated with one of four doses (1:0, 1:2.5, 1:10, 1:25) and a control adjuvant group. The most protective dose was then used as a marker of virulence in four groups (n=10; 4 vaccinated, 6 non-vaccinated) inoculated with four characterized *C. fetus* subsp. *venerealis* strains. The strains were selected based on biochemical and molecular profiles, with assistance from Dr Indjein in choice of those possessing genomic virulence markers of interest. Strain pathogenicity was monitored by assessing abortion rates as well as time to abortion, re-isolation of the causative bacterium by culture and/or detection by PCR, and histopathological markers of inflammation present in uterine and placental tissues.

Chapter 3. Chemicals, Media, Reagents **and Equipment**

3.1 Chemicals

AnalaR Normapur, VWR International Pty Ltd, Murrarie, QLD, Australia

EDTA (Ethylenediamine tetraacetic acid)

Glycine

Glycerol

Amyl Media, Rowe Scientific Pty Ltd, Dandenong, VIC, Australia

Bacteriological charcoal

Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia

Agarose

Tris

Invitrogen Australia Pty Ltd, Mulgrave, VIC, Australia

Sterile inactivated bovine serum

Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia

Amphotericin B

Bromophenol blue

Calcium chloride

Cyclohexamide

Disodium hydrogen phosphate

Ferrous sulphate

Magnesium sulphate

Nalidixic acid

Polymyxin B

Potassium chloride

Potassium dihydrogen phosphate

Sodium metabisulphite

Sodium pyruvate

Sodium chloride

Trimethoprim

Vancomycin

Yeast extract

3.2 Microbiological Media

Oxoid Australia, ThermoFisher Scientific Pty Ltd, Adelaide, SA, Australia

Bacteriological agar

Brain heart infusion (BHI)

Antimicrobial susceptibility discs

Ampicillin (AMP10)

Amox-Clavul (AMC30)

Apramycin (APR15)

Bacitracin (B0.05)

Cefuroxime (CXM30)

Ceftiofur (EFT30)

Cephalothin (KF30)

Co-trimoxazole (SXT25)

Enrofloxacin (ENR5)

Furazolidone (FR100)

Gentamicin (CN10)

Lincospectin (LS109)

Nalidixic acid (NA30)

Neomycin (N30)

Novobiocin (NV30)

Oxacillin (OX1)

Oxolinic acid (OA2)

Penicillin (P10)

Streptomycin (S10)

Sulphonamides (S3300)

Tetracycline (TE30)

Ticarcillin (TIC75)

Tilmicosin (TIL15)

Trimethoprim (W5)

CampyGen sachets

Campylobacter selective agar plates

Campylobacter selective supplement

Horse blood

Mueller-Hinton

Nutrient broth

Sheep blood agar plates

Triple-sugar iron slopes

Tryptone soya broth

Vegetable peptone no. 1

Fluka Analytical, Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia

Lead acetate paper

L-cysteine hydrochloride anhydrous

MP Biomedicals, Seven Hills, NSW, Australia

Sodium succinate

Remel, ThermoFisher Scientific Australia Pty Ltd, Adelaide, SA, Australia

McFarland turbidity equivalence standard 0.5

3.3 Laboratory prepared media

AQIS-compliant Campylobacter Isolation Medium

Private and confidential medium recipe obtained from Zoetis (Melbourne, Victoria, Australia)

Brain-heart Infusion (BHI) Broth

BHI	3.7 g
Yeast extract	0.2 g
Bacteriological agar no. 1	0.07 g
Milli-Q water	100 ml

Add reagents to water and boil to dissolve. Dispense in 10 ml volumes and autoclave at 121°C for 20 min. Store at 2-8°C for up to 3 months.

Campylobacter fetus Culture Media*

Private and confidential medium recipe obtained from Zoetis (Melbourne, Victoria, Australia)

*For biochemical tolerance media, *Campylobacter fetus* culture media may be modified by the addition of the following reagents in the first step:

1% glycine media – 1 g glycine

3.5% NaCl media – 1 g sodium chloride

H₂S detection media – 0.03 g cysteine-HCl

Campylobacter Growth Supplement

Sodium pyruvate	5 g
Sodium metabisulphite	5 g
Ferrous sulphate	5 g
Milli-Q water	80 ml

Dissolve reagents in water by heating to 56°C. Aseptically filter solution through a 0.2 µm filter and dispense into 4 ml aliquots. Store at -2 to -8°C until required.

FBP (ferrous sulphate, sodium bisulphite, sodium pyruvate) Storage Medium (Gorman and Adley 2004)

BHI or vegetable peptone no. 1	5 g
Bacteriological agar	0.24 g
Yeast extract	0.2 g
Glycerol	30 ml
Milli-Q water	200 ml
Growth supplement	4 ml

Dissolve BHI, agar and yeast extract in water by boiling in microwave on high in short bursts. Add glycerol and mix to disperse. Sterilize by autoclaving at 121°C for 30 min along with Wheaton vials for dispensing. Allow to cool to 56°C in a water bath before aseptically adding growth supplement. Dispense 150 µl media into Wheaton vials aseptically and store at 2-8°C until required.

Modified Lander's TEM (Hum, Hornitzky et al. 2009)

Base

Mueller-Hinton broth	21 g
Bacteriological charcoal	5 g
Milli-Q water	1000 ml

Add reagents to water and mix to disperse by placing on magnetic stirrer with stirrer bar. When charcoal is in suspension (will not fully dissolve) sterilize by autoclaving at 121°C for 20 min. Allow to cool to 56°C in a water bath for approximately 1 h.

Additives

Lysed horse blood	70 ml
Campylobacter growth supplement	4 ml
Cyclohexamide (0.01 g/ml)	10 ml
Vancomycin (0.004 g/ml)	5 ml
Trimethoprim (0.002 g/ml)	5 ml
Polymyxin B (100 000 IU/ml)	25 µl

Once the base has cooled, aseptically add all of the above additives. Dispense 5 ml media into sterile 9 ml culture tubes aseptically while stirring to avoid charcoal settling. Store at 2-8°C for up to 3 months.

Phosphate Buffered Saline (PBS)

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.44 g
KH ₂ PO ₄	0.24 g
Milli-Q water	1000 ml

Add all reagents to 900 ml water and mix to dissolve. Adjust to pH 7.4 using 1 M NaOH or HCl. Adjust the final volume to 1000 ml before dispensing in required volumes. Sterilize by autoclaving at 121°C for 20 min.

Thomann Transport and Enrichment Medium (TTE) (Harwood, Thomann et al. 2009)

Milli-Q water	1000 ml
Nutrient broth	25 g
Campylobacter growth supplement	4 ml
Skirrow's supplement	3 ml
Amphotericin B	5 mg

Add nutrient broth to water and mix to dissolve. Sterilize by autoclaving at 121°C for 30 min. Allow to cool to 56°C in a water bath before aseptically adding Campylobacter growth supplement Skirrow's supplement and amphotericin B. Dispense 5 ml media into sterile 9 ml culture tubes aseptically and store at 2-8°C until required.

3.4 Equipment and Software

Applied Biosystems, Foster City, California, USA

ABI7500 Fast Real-Time PCR system

Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia

DNA Engine[®] Thermal Cycler

GelDoc[™] XR+ system

Sub-Cell[®] Model 192 Agarose Gel Electrophoresis system

Corbett Life Science, Qiagen Pty Ltd, Chadstone, VIC, Australia

Corbett Rotor-Gene RG-3000

Department of Agriculture and Fisheries, Brisbane, QLD, Australia

Tricamper[™] sampling tool

Don Whitley Scientific Pty Ltd, North Gosford, NSW, Australia

Whitley DG250 Microaerophilic Workstation

Nikon, Tokyo, Japan

Nikon Eclipse E400 Microscope

Thermo Labsystems, ThermoFisher Scientific Pty Ltd, Adelaide, SA, Australia

Multiskan Spectrum Spectrophotometer

Multiskan Ascent Plate Reader

Esaote Pie Medical, Genoa, Italy

MyLab 30 Vet Ultrasound Scanner

Universal Vaporizer Support, Foster City, California, USA

Universal Vaporizer Anaesthetic machine

StataCorp LP, College Station, TX, USA

STATA statistical analysis software

Chapter 4. Evaluation of Transport and
Culture Methods for the Isolation of
Campylobacter fetus* subsp. *venerealis

4.1 Introduction

Diagnosis of BGC relies on the culture of *C. fetus* subsp. *venerealis* from submitted samples, while failure to isolate the organism can result in misdiagnosis or incomplete diagnosis. Isolation of the causative bacterium is recognisably unreliable (Clark 1971, Lander 1990, Monke, Love et al. 2002, Hum, Hornitzky et al. 2009). This is due to the fragile and fastidious nature of the bacteria requiring sensitive specific growth and atmospheric conditions for survival while often overgrown by ubiquitous contaminating organisms. Thus transport and enrichment media (TEMs) have been developed to support the growth of *C. fetus* subsp. *venerealis* while inhibiting contaminants (Clark, Monsbrough et al. 1974, Lander 1990, Hum, Hornitzky et al. 2009). Samples requiring prolonged transport time are often inoculated into a TEM prior to conveyance. In countries with extensive cattle production enterprises, such as Australia, samples sent to diagnostic labs over great distances (approximately 2 000 kilometres from northern Queensland to state diagnostic laboratories), often at variable temperatures (-10°C - 49°C), require an ideal TEM for the support and enrichment of the causative bacterium.

Phosphate buffered saline (PBS) is commonly used for the collection of samples not requiring extended transportation, however, for those samples necessitating greater than 6-8 h travel prior to reaching the laboratory, a number of TEMs are available (OIE 2012). These include; Clark's, Lander's (also known as modified Weybridge) as recommended by the World Organisation for Animal Health (OIE), as well as a more recently developed media that is able to be used for both culture and molecular testing; Thomann Transport and Enrichment (TTE) medium (Harwood, Thomann et al. 2009). Lander's has been modified by Hum (2009) to reduce the concentration of polymyxin B due to sensitivities shown by *C. fetus* subsp. *venerealis* strains (Jones, Davis et al. 1985, Hum, Hornitzky et al. 2009). No studies have compared the efficacy of TTE compared to Lander's yet it was reported that TTE increases the viability of *C. fetus* subsp. *venerealis* at temperatures ranging from 25°C to 37°C (Harwood, Thomann et al. 2009).

A variety of these TEMs (Clark's, Lander's, Weybridge) are used across laboratories and protocols with diversity in selective culture media (SBA, Campylobacter agar, Preston's agar, Skirrow's agar) (Hum and McInnes 1993, Monke, Love et al. 2002, Chaban, Guerra et al. 2013). The ideal medium should enrich *C. fetus* subsp. *venerealis*, while reducing the growth of other fast growing species of bacteria. The most common contaminants found in preputial samples appears to be *Pseudomonas* and *Proteus* species which are ubiquitous and often, due to their faster growing and swarming nature, overgrow *C. fetus* subsp. *venerealis* in TEMs. They have been shown to cover the entire

surface of inoculated culture media, inhibiting the growth and identification of *C. fetus* subsp. *venerealis* colonies (Samuelson and Winter 1966, Clark 1971, Monke, Love et al. 2002). Contamination of TEMs for the enrichment of *C. fetus* subsp. *venerealis* can also be caused by other bacteria including: *Escherichia coli*, *Corynebacterium*, *Micrococcus* and other non-pathogenic intestinal thermophilic *Campylobacter* spp. (Ruebke 1951, Lovell 1963) (Indjein 2013). For example ‘*V. bubulus*’ is a documented commensal bacterium commonly found in bull penile sheaths (Veron and Chatelain 1973). ‘*V. bubulus*’ has been shown to be limited to the anterior prepuce in bulls, while *C. fetus* subsp. *venerealis* is commonly found in the fornix (Samuelson and Winter 1966). Both species are carried by bulls asymptotically with one study demonstrating their presence in 55.6% bulls examined in the United States (Samuelson and Winter 1966). The feature assumed to account for the presence of *C. fetus* subsp. *venerealis* and ‘*V. bubulus*’ at these sites is believed to be the microaerobic atmosphere provided by the preputial crypts (Samuelson and Winter 1966), however, other site specificities including temperature or pH have not been studied. In heifers, the infection is believed to localize in the anterior vagina and cervix, ascending into the uterus and oviducts (Clark 1971). A recent Australian study showed that isolation of *C. fetus* subsp. *venerealis* can often be confused by the presence of *C. hyointestinalis*, *C. ureolyticus* and *Arcobacter* species (Nordin 2013). Only 39.5% of the 43 *Campylobacter*-like isolates from bull penile sheaths identified phenotypically as *C. fetus* subsp. *venerealis* were confirmed by heat shock protein gene (*cpn60*) sequence analysis (Nordin 2013). The remaining isolates consisted of predominantly *Arcobacter* species (20.9%), *C. hyointestinalis* (13.9%), unidentifiable bacteria (11.7%), *C. ureolyticus* (4.7%), *C. sputorum* (4.7%) and non-*Campylobacter* species (4.7%) (Nordin 2013).

Different microbial flora in heifers, which have been shown to commonly consist of *micrococci* or *streptococci* in the USA, are less likely to produce spreading coverage of culture media (Dawson 1959). The technique used for collection of samples can also impact on the isolation results, with a study conducted in Uruguay comparing scraping, aspiration and washing finding 71.4%, 36.0% and 25.8% success, respectively. In the same study it was shown that the incidence of high level contamination appeared to also correlate with the sampling method with 33.9%, 63.0% and 83.9% seen with scraping, aspiration and washing, respectively (Tedesco, Errico et al. 1977). While the OIE and ANZSDP both include all options as part of recommended procedure, it is stated that scraping is the most sensitive method (Hum, Hornitzky et al. 2009, OIE 2012). This is supported in the literature with many studies, both in Australia and internationally using scraping for the

collection of samples (Hum, Brunner et al. 1994, Chaban, Guerra et al. 2013, Indjein 2013, Guerra, Chaban et al. 2014).

Inhibition of these commonly present bacteria to allow isolation of *C. fetus* subsp. *venerealis* has found that size or antimicrobial exclusions may assist with the reduction of overgrowth on culture media (Lovell 1963, Hum, Brunner et al. 1994, Monke, Love et al. 2002, Chaban, Guerra et al. 2013). A number of selective agars for the isolation of *Campylobacter* species are available. Most commonly, Skirrow's agar is recommended for the isolation of *C. coli* and *C. jejuni* containing a combination of antibiotics; trimethoprim, vancomycin, amphotericin B and polymyxin B (Oxoid, ThermoFisher Scientific Australia, Adelaide, SA, Australia). Preston's *Campylobacter* agar recommended for general isolation of *Campylobacter* species contains trimethoprim, rifampicin, cyclohexamide and polymyxin B (Oxoid). Other selective non-blood base charcoal agars are available and are used in various diagnostic laboratories. A different combination of antibiotics included bacitracin, novobiocin, polymyxin B and cyclohexamide as used by Dufty (Dufty 1967) was compared in an Australian study with Skirrow's agar on 275 samples from 176 animals (Hum, Brunner et al. 1994). This included samples from a single persistently infected bull that was sampled by aspiration, 145 bulls sampled at abattoir using scraping and 30 heifers sampled by swabbing the cervico-vaginal mucus. Samples were immediately inoculated onto agar, as well as placed into normal saline. The saline solution was then used to inoculate two different TEMs, Clark's and Lander's and incubated for 72 h before subculturing onto the two different agars. Nineteen samples were found to be positive overall, of which 98.7% were detected using Dufty's selective agar, while only 68.4% were isolated on Skirrow's agar by direct culture. Comparison of the two TEMs found that Lander's performed better than Clark's with 73.7% of the positive cultures detected on both agars, with only 26.2% detected using Clark's (Hum, Brunner et al. 1994). A Canadian study compared the efficacy of Skirrow's selective agar with non-selective blood agar from 217 samples collected from naturally (n=4) and artificially infected (n=8) bulls. Samples were collected using an insemination pipette into PBS which was used to inoculate Lander's TEM. After 72 h incubation Lander's was subcultured onto Skirrow's and blood agar. Skirrow's agar resulted in 40% positive isolations compared to 35% with blood agar (Chaban, Guerra et al. 2013). Selective agars are limited in their ability to preclude contaminants based on the antibiotics contained within them. This is restricted to antibiotics and concentrations that do not inhibit the growth of *C. fetus* subsp. *venerealis*.

Filtration as a method of size exclusion has been used in numerous studies for the isolation of different *Campylobacter* species in human and veterinary diagnostic labs from intestinal contents, blood and faecal matter (Plumer, Duvall et al. 1962, Lastovica and le Roux 2000, Kulkarni, Lever et al. 2002, Speegle, Miller et al. 2009, Chaban, Guerra et al. 2013). Variations in filter size have been researched, with 0.65 µm filters more commonly used than 0.45 µm filters (Lastovica and le Roux 2000, Speegle, Miller et al. 2009, Chaban, Guerra et al. 2013). However, a comparative study found that while the use of a 0.65 µm filter produced more *Campylobacter* cultures, it also was unable to prevent overgrowth of 40% of contaminated samples (Plumer, Duvall et al. 1962). Scanning electron microscopy of broiler samples was able to show that 15 min was sufficient to allow *C. jejuni* and *C. coli* to pass through the filter onto the agar below (Speegle, Miller et al. 2009), while up to half an hour has allowed for detection of common enteropathogenic *Campylobacter* species as well as *Arcobacter* (Kulkarni, Lever et al. 2002). Studies have also produced contending results as to whether filter or antimicrobial selectivity is preferential for improved culture success, with a study showing an increase from 7.1% culture rate using Skirrow's agar to 21.8% using 0.65 µm filtration on non-selective agar (Lastovica and le Roux 2000). A second study found that molecular screening of faecal samples produced 20 *Campylobacter* positives including *C. coli*, *C. jejuni*, *C. hyointestinalis* and *Arcobacter*, of which selective agar was able to detect 17 samples, while filtration onto non-selective blood agar detected only 12 samples (Kulkarni, Lever et al. 2002). The most recent study has shown that 0.65 µm filtration of bull preputial samples collected in PBS onto non-selective blood agar was significantly better than selective culture on Skirrow's agar with 86.5% and 32.0% of positive *C. fetus* subsp. *venerealis* cultures detected respectively (Chaban, Guerra et al. 2013). Failure to isolate *C. fetus* subsp. *venerealis* can be due to a number of different factors, including inappropriate collection or culture methods. This could be due to sampling and/or TEMs not adequately enriching or sustaining the bacteria or sufficiently hindering contaminants. It may also be due to culture media allowing overgrowth of contamination or precluding *Campylobacter* colonies due to size or antimicrobial susceptibilities.

While molecular testing is believed to be more sensitive than culture, there is currently a lack of specific and sensitive assays to aid with diagnosis of BGC. Commonly used assays include the *parA* (McMillen, Fordyce et al. 2006) and *cstA/parA* duplex PCR (Hum, Quinn et al. 1997) which have had questionable specificity. Recent findings have shown that the *parA* PCR often used for confirmatory testing of isolated strains has a 53% sensitivity for *C. fetus* subsp. *venerealis* strains while the duplex PCR was marginally more sensitive at 58% (van der Graaf-van Bloois, van Bergen et al. 2013).

Therefore, this chapter aimed to evaluate the ability of two different TEMs; OIE recommended Lander's and the newer TTE, and two selective culture media (sheep blood agar with and without 0.45 µm filtration and Campylobacter agar) to enrich and isolate *C. fetus* subsp. *venerealis* while restricting the growth of common contaminants. This will be done by comparing revival and re-isolation of stored, contaminated *C. fetus* subsp. *venerealis* isolates by direct plating onto non-selective sheep blood agar (SBA) with and without 0.45 µm filtration vs. selective agar (Campylobacter). Secondly, through a laboratory trial comparing the ability of TTE and Lander's to sustain *C. fetus* subsp. *venerealis* growth independently and in the presence of one of three different contaminant bacteria (*P. aeruginosa*, *P. vulgaris* or *C. jejuni*) under two incubation temperatures (25°C and 42°C) for up to 7 days. And lastly, using a cattle infection trial of heifers (n=28) and bulls (n=28) experimentally inoculated with one of two *C. fetus* subsp. *venerealis* strains to compare the success rates of re-isolation when using TTE, Lander's and PBS as transport media over a 6 week period after inoculation.

4.2 Material and Methods

4.2.1 Media

All media used for the growth and testing of the strains in this chapter have been outlined in Chapter 3. These include laboratory made media; tryptone-soya broth and Campylobacter broth for the cultivation of strains, 0.02% cysteine media and 1% glycine media for biochemical tolerance testing and TTE, Lander's and PBS for transport and enrichment. Other commercially purchased media and reagents include SBA (Oxoid), Campylobacter agar (Oxoid), oxidase (BD), TSI slopes (Oxoid), nalidixic acid, cephalothin, tetracycline and streptomycin antimicrobial susceptibility disks (Oxoid).

4.2.2 Bacterial strains

C. fetus subsp. *venerealis*

Two biovars of *C. fetus* subsp. *venerealis* (Strains 76223 and 924) were selected for inoculation of 56 animals in a large animal infection trial carried out at the University of Queensland farm at Pinjarra Hills, SE QLD (27.4667° S, 153.0333° E) in February/March 2012. Strain 76223 was isolated at the Biosecurity Queensland laboratory from a cattle abortion event in South-East QLD and was assumed to be pathogenic. Strain 924 was isolated from a bull prepuce abattoir study (Indjein 2013). Strain 924 displayed resistance to tetracycline and streptomycin antibiotics in laboratory testing which were of interest given findings of pathogenicity islands situated on *C. fetus* genes associated with antibiotic resistance (Abril, Brodard et al. 2010).

The two strains were resuscitated from storage at -80°C and inoculated onto SBA (Oxoid) and Campylobacter agar (Oxoid) plates. These were incubated microaerobically at 37°C for 48 - 72 h and checked for pure growth. An individual, isolated colony was picked using a disposable 10 µl loop and placed into Campylobacter broth. These broths were incubated at 37°C microaerobically for 48 - 72 h. Strains were then molecularly and biochemically profiled as stipulated in the OIE guidelines (OIE 2012). Phenotypic tests included oxidase, catalase, growth at 25°C and 42°C, production of H₂S in a cysteine broth or triple sugar iron (TSI) slope, growth in 1% glycine and antibiotic results for nalidixic acid, cephalothin, tetracycline and streptomycin.

Strain 76223 was subsequently sequenced at Biosecurity Queensland laboratory using universal primers for the bacterial 16S ribosomal RNA gene resulting in a 100% sequence similarity with *C. fetus* subsp. *venerealis* and confirmed by MLST typing (results not shown) (Nordin 2013).

Table 6. Biochemical characteristics of two *C. fetus* subsp. *venerealis* strains (76223 and 924) used for inoculation of 56 bulls and heifers in Experiment 3 according to OIE guidelines (OIE 2012).

Strain	Oxidase	Catalase	25°C	42°C	H ₂ S TSI	H ₂ S broth	1% Glycine	Nalidixic acid	Cephalothin	Tetracycline	Streptomycin
76223	+	+	+	-	-	-	-	S	S	S	S
924	+	+	+	-	-	+	-	R	R	R	R

TSI – triple sugar iron; S – sensitive; R - resistant

4.2.3 Other Bacterial Strains

Pseudomonas and *Proteus*

Pseudomonas aeruginosa strain ATCC 25922 and *Proteus vulgaris* strain ATCC 6380 were used and confirmed phenotypically by Biosecurity Queensland laboratory. The strains were resuscitated from storage at -80°C and inoculated onto SBA plates (Oxoid). These were incubated aerobically at 37°C for 24 h and checked for pure growth. An individual, isolated colony was picked using a disposable 10 µl loop and placed into tryptone-soya broth (Chapter 3). The broth was incubated at 37°C aerobically until the culture reached mid-log phase at OD₆₀₀=0.5 optical density (Multiskan Spectrum, Thermo Labsystems, ThermoFisher Scientific, Adelaide, SA, Australia) which was previously identified to be approximately 10⁵ CFU/ml.

Thermophilic *Campylobacter*

Campylobacter jejuni strain ATCC 33291 was used and confirmed phenotypically by the Biosecurity Queensland laboratory. The strain was resuscitated from storage at -80°C and inoculated onto an SBA and *Campylobacter* agar plate. This was incubated microaerobically at 37°C for 48 - 72 h and checked for pure growth. An individual, isolated colony was picked using a disposable 10 µl loop and placed into *Campylobacter* broth. This was incubated at 37°C in the microaerobic workstation (Don Whitley Scientific) for 48 – 72 h. At 48 and 72 h, a serial dilution of 10⁻¹ to 10⁻⁹ CFU/ml was prepared and plated in duplicate by placing 100 µl of the dilution onto an SBA plate and spreading it evenly across the surface. The plates were then incubated in the microaerobic workstation for 72 h. Subsequently, a comparative cell count using a Helber bacterial counting chamber (Hawksley, Sussex, UK) was carried out by creating a 1/10 dilution of the broth in 10% neutral buffered formalin (NBF) (10% w/v formaldehyde, Merck Australia, Merck KGaA, Germany) to fix the bacteria. A volume of 10 µl was placed into the central chamber of the counter,

the coverslip added and viewed under a Nikon Eclipse E400 microscope (Nikon, Tokyo) at 400 x magnification and the concentration calculated (Meynell and Meynell 1970). The bacteria were diluted to 1×10^5 CFU/ml for subsequent mixed culture experiments.

4.2.4 Transport and Enrichment Media (TEM)

Three TEMs were tested; Lander's (Hum, Hornitzky et al. 2009), Thomann Transport and Enrichment (TTE) media (Harwood, Thomann et al. 2009), and Phosphate Buffered Saline (PBS) (OIE 2012). TEMs were incubated under aerobic conditions at 37°C or 25°C as required.

4.2.5 Selective Culture Media and Incubation

Two different agar plates were used, a non-selective 5% SBA plate and *Campylobacter* agar. SBA plates were used with and without a 0.45 µm filter. All plates were incubated at 37°C for 72 h in a microaerobic chamber (Don Whitley Scientific, North Gosford, NSW, Australia) with a commercially provided gas mixture (5% O₂, 10% CO₂, 85% N).

Experiment 1: Resuscitation of contaminated stored *C. fetus* subsp. *venerealis* isolates

All *C. fetus* subsp. *venerealis* isolates used in this chapter were isolated and stored in 85% glycerol or 15% FBP at -80°C as part of a previous study (Indjein 2013). A number of these isolates were resuscitated to allow biochemical characterisation (Chapter 5) and for use in animal model research (Chapter 4 Experiment 3, and Chapters 6 and 7). Upon resuscitation, it was noted that a subset (n=31), of isolated and stored samples (n=84) between December 2009 and October 2010 as part of a parallel PhD project were contaminated and failed to yield any *Campylobacter* colonies by normal resuscitation methods i.e. 100 µl of thawed stored broth inoculated into 10 ml of *Campylobacter* broth (Chapter 3) followed by subculture onto an SBA plate after 48 – 72 h or directly onto an SBA plate and streaked out. These were incubated microaerobically at 37°C for 48 – 72 h and checked for growth; broth turbidity or colonies on agar.

Due to the shortage of available *Campylobacter* isolates as well as the usefulness of these contaminated strains in identifying the most suitable methodologies for isolation, they were used in this experiment, with confirmation of isolates biochemically and molecularly. As a result of overgrowth of contaminating organisms in stored isolates, a modified method was implemented for the resuscitation of these 31 isolates and this contamination storage issue provided an opportunity to test filtration which was not routinely implemented at the Biosecurity diagnostic laboratory previously. This included direct inoculation of 200 µl of thawed storage broth onto a 0.45 µm filter

placed aseptically onto the surface of an SBA plate before removing the filter after 15 mins. Alternatively 100 µl of thawed storage broth was inoculated into 10 ml of Campylobacter broth and incubated microaerobically at 37°C for 48 – 72 h followed by subculture onto an SBA plate with a 0.45 µm filter and a Campylobacter agar plate. Culture media was incubated as above for up to 7 d. All *Campylobacter*-like isolates were re-streaked from a single colony to produce a pure culture, which was then biochemically profiled to confirm *C. fetus* subsp. *venerealis* identity using OIE recommended tests. These included oxidase, catalase, growth at 25°C and 42°C, production of H₂S in a cysteine broth or triple sugar iron (TSI) slope, growth in 1% glycine and antibiotic results for nalidixic acid, cephalothin, tetracycline and streptomycin.

Experiment 2: Laboratory simulation for the evaluation of *C. fetus* subsp. *venerealis* transport media and isolation methods

An experiment was conducted to determine the viability of *C. fetus* subsp. *venerealis* in Lander's TEM or TTE media, independently and in the presence of common contaminating organisms. A preliminary experiment was conducted to determine the concentration of *C. fetus* subsp. *venerealis* required in the media to allow isolation after 24 h incubation using filtration. A reference strain (ATCC 19438) of *C. fetus* subsp. *venerealis* in Lander's medium was grown for 72 h in the microaerobic chamber. At 72 h the concentration was calculated as in section 4.2.3. A serial dilution of this medium was created into a further 7 tubes of Lander's media. These dilutions were incubated microaerobically and plated out daily for 7 d onto SBA, SBA with a 0.45 µm filter and Campylobacter agar plates. Plates were incubated in the microaerobic station and checked at 48 – 72 h for growth.

The effect of contaminating organisms on *C. fetus* subsp. *venerealis* growth in the two different media was assessed. This was done by spiking 40 tubes for both TTE and Lander's with 100 µl of 1×10^5 CFU/ml of *C. fetus* subsp. *venerealis* (strain 76223), a concentration believed to be at the higher end of that present in bull penile sheaths (Clark 1971). Groups of 10 of these tubes were inoculated with one of the following species of bacteria: *P. aeruginosa*; 10 µl at mid-log phase, *P. vulgaris*; 10 µl at mid-log phase, *Campylobacter jejuni* (100 µl of 1×10^5 CFU/ml) and ten tubes containing only *C. fetus* subsp. *venerealis* (Figure 3). Half of each set of ten tubes was incubated at 37°C (5 replicates) as is suggested in OIE guidelines (OIE 2012), while the other half (5 replicates) was incubated at 25°C as a differential selective process to favour growth *C. fetus* subsp. *venerealis*. The TEM tubes were plated at 24, 48, 72 and 168 h onto culture media consisting of 10 µl streaked on an SBA plate, 200 µl transferred onto a SBA plate with a 0.45 µm filter and allowed to stand for

approximately 15 min, and 10 µl streaked onto a Campylobacter agar plate. Plates were then placed into a 37°C microaerobic chamber and checked at 48 - 72 h for growth.

Growth of *C. fetus* subsp. *venerealis* was scored in relation to the contaminant (if present), with light growth (<10 colonies), moderate growth (10 – 50 colonies) and heavy growth (>50 colonies) while the contaminating organism was scored as light growth (<10 colonies), moderate growth (1/3 of the plate), heavy growth (2/3 of the plate) and complete (3/3 of the plate) (Indjein 2013). All *Campylobacter*-like isolates were re-streaked to obtain a pure culture, which was then biochemically profiled (section 2.2.1) to determine correspondence with spiked bacteria.

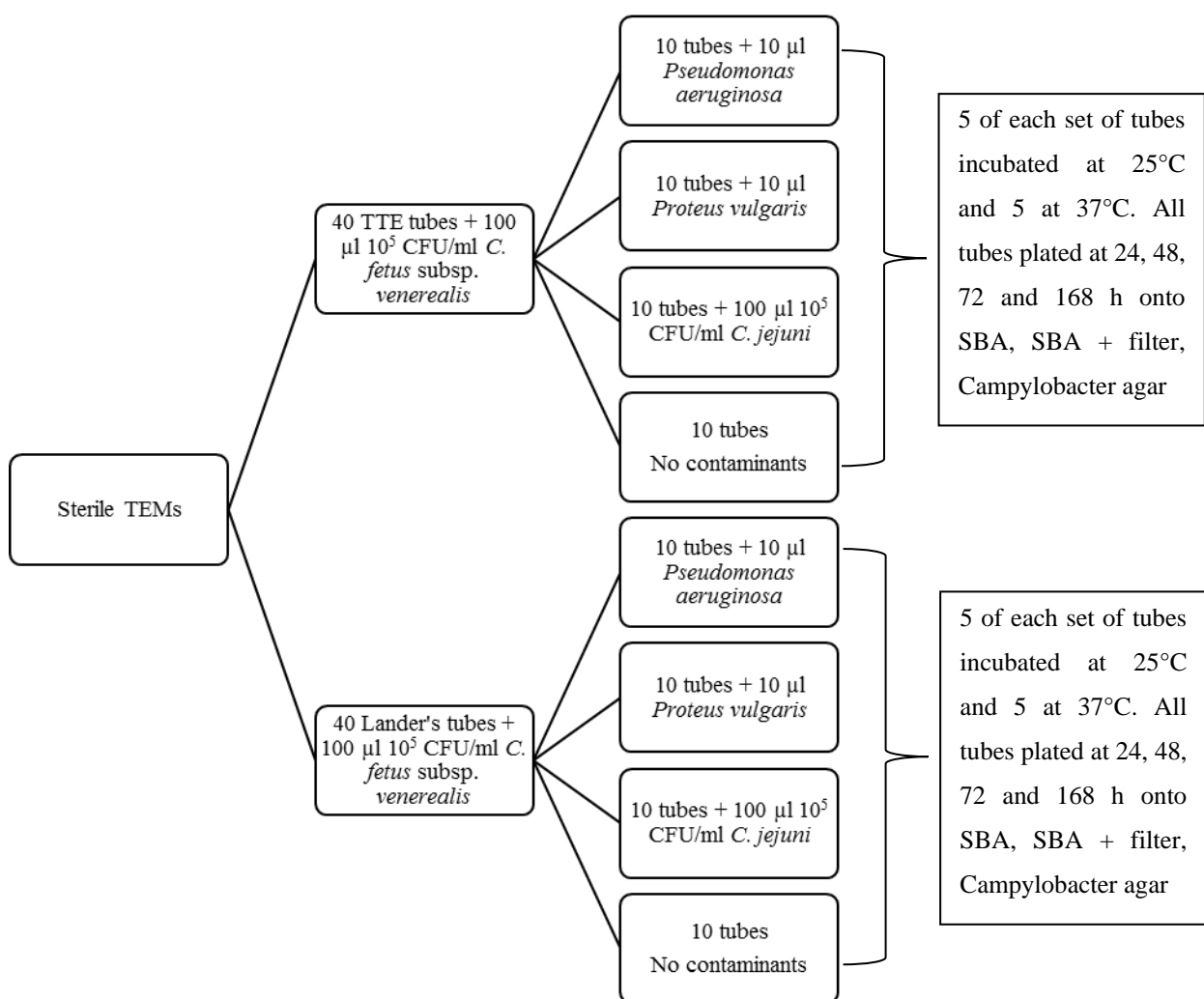


Figure 3. Schematic representation of Experiment 2 showing the inoculation of 40 tubes each of two different transport and enrichment media (TEM); Lander's and TTE with *C. fetus* subsp. *venerealis* strain 76223 followed by addition of one of three contaminating organisms to groups of 10 tubes, *P. aeruginosa*, *P. vulgaris* or *C. jejuni*. Half the tubes were differentially incubated at 25°C or 37°C

with culture at 24, 48, 72 and 168 hours (h) via three methods; directly onto sheep blood agar (SBA), onto a 0.45 µm filter on SBA and directly onto Campylobacter agar.

Experiment 3: *In vivo* evaluation of the two TEMs (Lander's and TTE)

The two selected *C. fetus* subsp. *venerealis* strains (76223 and 924) were each inoculated into 10 ml of fresh vegetable peptone based Campylobacter broth (Chapter 3) and incubated in a microaerobic workstation at 37°C for 72 h. The bacterial concentration was calculated as in section 4.2.3 at 72 h. Inoculum were streaked out just prior to inoculation onto two SBA plates, incubated at 37°C either in the microaerobic workstation or in an aerobic incubator to ensure sterility of the broth.

A total of 56 animals were inoculated, 28 inoculated with strain 76223 (n=14 bulls and n=14 heifers) and the remaining 28 inoculated with strain 924 (n=14 bulls and n= 14 heifers). All animals used in the study were less than three years of age. Semen and vaginal mucus samples from all animals were tested using the duplex *cstA/parA* PCR and the *parA* real time PCR (Hum, Quinn et al. 1997, McMillen, Fordyce et al. 2006) and by culture weekly for three weeks prior to inoculation, and were shown to be free from infection with *C. fetus* subsp. *venerealis*. Inoculation of bulls was conducted on four successive days to establish infection using a sterile artificial insemination pipette (Bovivet®) with 5 ml of inoculum between $10^7 - 10^8$ CFU/ml per dose. The preputial orifice was closed using digital pressure post-inoculation and the prepuce externally massaged for 2-3 min. Heifers were oestrus synchronized (injectable program) and inoculation was carried out on four successive days. The first inoculation (day 0) was conducted at oestrus with concurrent insemination using fresh semen from confirmed BGC negative bulls. An equivalent volume and concentration of inoculum was used to inoculate heifers via an artificial insemination pipette intra-cervically. The heifers and bulls were kept at different locations within the farm. Vaccinated and non-vaccinated animals were kept together and procedures were done at different handling facilities so the risk of cross-contamination was reduced. Further details with respect to the animal trial is outside the scope of this PhD and is part of a concurrent PhD project by Andres Ardila Avila (School of Veterinary Science UQ).

Animals were sampled for culture and PCR once a week over a further 6 weeks, with bulls not sampled at week 1 due to adverse weather. The animals were tested by culture to determine infection status. Smegma or vaginal mucus scrapings were collected in PBS, Lander's and TTE using three different Tricampers™ to allow each one to be cut off directly into each respective medium. While processing animals, samples collected remained at ambient temperature. TEM

inoculated with Tricampers™ were brought back to the laboratory (within 4-6 h of collection) and immediately plated onto the three culture media: SBA, SBA with a 0.45µm filter and Campylobacter agar as described above. Plates were incubated in the microaerobic chamber at 37°C for 72 h and checked daily. The TEMs were then incubated at 37°C for 24 h and plated onto the same three culture media. All *Campylobacter*-like isolates which were those able to grow microaerobically, had a similar pink-grey colony morphology, displayed the characteristic spiral rod with corkscrew motility and were oxidase positive and Gram negative were re-streaked to produce a pure culture. These pure cultures were then biochemically profiled (section 4.2.2) to determine correlation with inoculated *C. fetus* subsp. *venerealis* strains. Differences in media and animals was analysed using STATA (StataCorp LP, College Station, TX, USA) with Fisher's exact test for comparisons.

4.3 Results

Experiment 1: Resuscitation of contaminated stored *C. fetus* subsp. *venerealis* isolates

Recovery of contaminated stored isolates was significantly more successful from direct filtration onto an SBA plate with 61% (19/31) of samples re-isolated (Table 2). Nine of these strains were only recovered by this method, with the remaining 10 samples were also recovered by either of the two remaining methods; enrichment in Campylobacter broth before culture onto SBA with a 0.45 µm filter or Campylobacter agar. All recovered strains were biochemically confirmed as *C. fetus* subsp. *venerealis*.

Table 7. Success rates of *C. fetus* subsp. *venerealis* recovery from contaminated storage broths at resuscitation from -80°C using four different culture methods; direct inoculation of storage medium onto SBA, SBA with a 0.45 µm filter, enrichment of storage broth in Campylobacter agar followed by either culture onto SBA with 0.45 µm filter or onto Campylobacter agar.

	Direct inoculation of SBA	Direct inoculation of SBA + 0.45 µm filter	Inoculation of Campylobacter broth followed by SBA + 0.45 µm filter culture	Inoculation of Campylobacter broth followed by Campylobacter agar culture
Resuscitated positive <i>Campylobacter</i> cultures	0/31 (0%)	19/31 (61%)	6/31 (19%)	8/31 (26%)

Experiment 2: Laboratory simulation for the evaluation of *C. fetus* subsp. *venerealis* transport media and isolation methods

The initial concentration of the reference strain (ATCC 19438) tested in Lander's was 5×10^7 CFU/ml. Therefore, the serial dilution produced concentrations of $0 - 10^6$ CFU/ml. Campylobacter agar and direct plating onto SBA were able to detect up to 10^2 CFU/ml after 24 h incubation while SBA with filtration could only detect up to 10^5 CFU/ml after 24 h. Optimal culture rates were reached at 72 h incubation of Lander's when all plating methods were able to detect the lowest dilution. At day 6 filtration results were intermittent over the dilutions with direct plating still producing positive results across all dilutions.

Mixed spiked cultures of both TTE and Lander's with *P. aeruginosa* under 25°C or 37°C incubation, regardless of culture media or incubation time failed to yield any *C. fetus* subsp. *venerealis* colonies (Table 8). *P. vulgaris*-inoculated Lander's and TTE did not return any *C. fetus*

subsp. *venerealis* cultures directly onto SBA plates or onto SBA with a 0.45 µm filter from TTE. Early culture media inoculations (24 - 48 h) from Lander's incubated at 25°C onto SBA with a filter produced low growth of *C. fetus* subsp. *venerealis* (<10 colonies). Campylobacter agar produced the highest recovery rate in the presence of *P. vulgaris* with isolations after 48 h incubation of TTE at 25°C and 72 h incubation from both TEMs at both 25°C and 37°C. The colony numbers appeared slightly higher from TTE, but both TEMs were able to sustain growth of *C. fetus* subsp. *venerealis* in the presence of the spiked bacteria for up to 7 days (Table 8).

Simulated mixed culture of *C. fetus* subsp. *venerealis* with the thermophilic *Campylobacter*, *C. jejuni* found that differential incubation at 25°C favoured *C. fetus* subsp. *venerealis* isolation from both TEMs and all culture media. At 37°C incubation, *C. fetus* subsp. *venerealis* was isolated only on SBA from Lander's with ≤72 h incubation and from TTE on SBA and Campylobacter agar, but only within 48 h incubation of TEM.

The use of a filter on SBA media was only useful in the isolation of *C. fetus* subsp. *venerealis* and *P. vulgaris* mixed cultures after 24 h incubation of Lander's. All other positive isolations were also positive on either SBA or Campylobacter agar and with higher colony counts of *C. fetus* subsp. *venerealis* (Table 8).

Peak concentrations of *C. fetus* subsp. *venerealis* were generally seen within 48 h incubation of both TEMs at either temperature. After 7 days incubation of both TEMs, the concentration of viable bacteria had decreased considerably in pure spiked TEMs. There did not appear to be a difference between the ability of the two TEMs to sustain *C. fetus* subsp. *venerealis* growth for 7 days, with similar growth on all culture media (Table 8).

Differential incubation of TEMs at 25°C compared to 37°C did not negatively impact on the recovery rate of *C. fetus* subsp. *venerealis*, and only marginally decreased the resultant colony numbers in two pure spiked cultures; Lander's after 24 h incubation on SBA and TTE after 72 h incubation on Campylobacter agar. Incubation at 25°C did however produce a higher number of isolations and colony counts of *C. fetus* subsp. *venerealis* cultures from thermophilic *Campylobacter* and *P. vulgaris* mixed cultures.

Table 8. Comparison of two transport and enrichment media (TEM); Landers and TTE and three culture media for isolation of *C. fetus* subsp. *venerealis* from spiked mixed cultures:

Sheep Blood Agar (SBA), SBA + 0.45 µm filter, Campylobacter agar after four different TEM incubation times; 24, 48, 72 and 168 h at two different temperatures; 25°C and 37°C. All media spiked with pure *C. fetus* subsp. *venerealis* (strain 76223) followed by inoculation with one of three organisms; *P. aeruginosa*, *P. vulgaris* or *C. jejuni*. Recovery recorded for *C. fetus* subsp. *venerealis* as – (no growth), + (light, <10 colonies), ++ (moderate, 10-50 colonies), +++ (heavy, >50 colonies). Growth of spiked bacteria recorded as; 0 – no growth, 1 - light, <10 colonies, 2 - moderate, 1/3 plate, 3 - heavy, 2/3 plate, and 4 - complete, 100% of plate.

TEM Media	Cultures	Incubation Temperature	Hours of TEM incubation prior to plating onto culture media											
			Sheep Blood Agar (SBA)				SBA + 0.45 µm filter				Campylobacter agar			
			24	48	72	168	24	48	72	168	24	48	72	168
Lander's	<i>P. aeruginosa</i> and 10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	3-	4-	4-	4-	0-	0-	0-	0-	3-	3-	4-	4-
		37°C	4-	4-	4-	4-	0-	0-	0-	0-	3-	3-	4-	4-
	<i>P. vulgaris</i> and 10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	3-	4-	4-	4-	0+	0+	0-	0-	3-	3-	2++	2+
		37°C	3-	4-	4-	4-	0-	0-	0-	0-	4-	4-	2++	2+
	<i>C. jejuni</i> and 10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	2++	2+++	0+++	0++	0-	0-	0+	0+	2++	2++	0+++	0++
		37°C	3+	3++	3+	3-	1-	1-	1-	1-	3-	3-	3-	3-
	10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	++	+++	+++	+	-	+	+	+	++	+++	+++	+
		37°C	+++	+++	+++	+	-	+	+	+	++	+++	+++	+
Thomann Transport and Enrichment (TTE)	<i>P. aeruginosa</i> and 10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	3-	4-	4-	4-	0-	0-	0-	0-	3-	3-	4-	4-
		37°C	3-	4-	4-	4-	0-	0-	0-	0-	3-	3-	4-	4-
	<i>P. vulgaris</i> and 10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	3-	4-	4-	4-	0-	0-	0-	0-	3-	3+	2++	2++
		37°C	4-	4-	4-	4-	0-	0-	0-	0-	4-	4-	2++	2++
	<i>C. jejuni</i> and 10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	0+	0++	0+++	0+++	0+	0+	0+	0+	2++	2+++	2+++	2++
		37°C	2-	2+	3-	2-	1-	1-	1-	1-	3+	3++	4-	4-
	10 ⁵ CFU/ml <i>C. fetus</i> subsp. <i>venerealis</i>	25°C	++	+++	++	+	+	+	+	+	++	+++	++	+
		37°C	++	+++	++	+	+	+	+	+	++	+++	+++	+

Experiment 3: *In vivo* evaluation of the two TEMs (Lander's and TTE)

Overall, there appeared to be a higher level of *Campylobacter*-like colony isolations from heifers when collected in Lander's media with 68 isolations over 6 weeks from a potential 168 samples with a spike at week 2 with 24 positive samples (n=28) as shown in Figure 4. For the bull cohort, there were a higher number of isolations within the 5 weeks, with 122 positive samples (n=504) (Table 9). Confirmation of these *Campylobacter*-like isolations according to OIE guidelines as *C. fetus* subsp. *venerealis* identified 97.1% positives from heifers, while only 41.8% of bull isolates matched the OIE biochemical profile. Overall, while TTE produced the highest level of *Campylobacter*-like isolations, 36.7%, the highest confirmed *C. fetus* subsp. *venerealis* isolations were from Lander's TEM with 26.0%. The difference in the detection of either *Campylobacter*-like organisms or confirmed *C. fetus* subsp. *venerealis* isolates from TTE vs. Lander's was not significantly different ($p = 0.1046$ and $p = 0.1037$, respectively), however, was significantly different between isolations from PBS compared to either TTE or Lander's ($p = 0.0001$). There was a significant difference in isolation success between bulls and heifers for either *Campylobacter*-like organisms or biochemically confirmed *C. fetus* subsp. *venerealis* ($p = 0.0020$ and $p = 0.00023$ respectively), but not when accounting for the lack of samples from bulls in week 1 and disregarding these results from for the heifers ($p = 0.0624$). The increased isolation of *Campylobacter*-like colonies from TTE compared to Lander's was significantly different ($p=0.0001$). According to biochemical characterisation, a total of 24 isolations produced H₂S positive results, although of these, only 12 matched animals which had in fact been inoculated with strain 924 which possessed this phenotype (Table 6). The remaining 200 isolations were all negative for H₂S production. Of all *Campylobacter* isolations, 47.8% were from animals inoculated with strain 76223 and 52.2% from those inoculated with strain 924. Seventy-two isolates (61.5%) from animals inoculated with strain 924 were H₂S negative, while 12 isolates (11.2%) from animals inoculated with strain 76223 were H₂S positive.

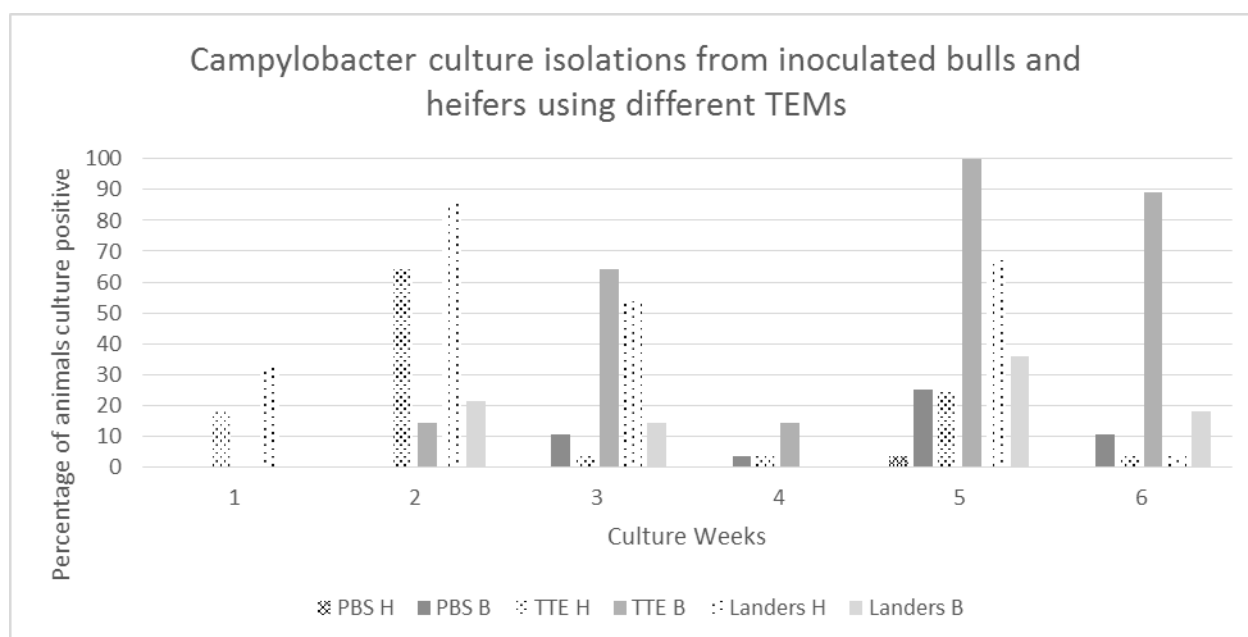


Figure 4. Percentage of inoculated bulls (n=28) and heifers (n=28) positive for *Campylobacter* culture from either of three tested Transport and Enrichment Media (TEM) over 5 - 6 weekly samplings (bulls not sampled at week 1). PBS H – *Campylobacter* isolations from heifer vaginal mucus samples collected in phosphate buffered saline; TTE H - *Campylobacter* isolations from heifer vaginal mucus samples collected in Thomann Transport and Enrichment medium; Lander's H - *Campylobacter* isolations from heifer vaginal mucus samples collected in Lander's medium; PBS B - *Campylobacter* isolations from bull preputial samples collected in phosphate buffered saline; TTE B - *Campylobacter* isolations from bull preputial samples collected in Thomann Transport and Enrichment medium; Lander's B - *Campylobacter* isolations from bull preputial samples collected in Lander's medium.

Table 9. Total number of samples from *C. fetus* subsp. *venerealis* strain 924 and 76223 inoculated animals (28 bulls and 28 heifers) that were culture positive during the 6 week period after inoculation for *Campylobacter*-like isolates and the number of these biochemically confirmed as *C. fetus* subsp. *venerealis* from either of the three tested Transport and Enrichment Media.

Isolations	Group	PBS	Lander's TEM	TTE media	Group Totals
Campylobacter-like*	Heifers	1/168 (0.6%)	68/168 (40.5%)	33/168 (19.6%)	102/504 (20.2%)
	Bulls	17/140 (12.1%)	25/140 (17.9%)	80/140 (57.1%)	122/420 (29.0%)
Media Totals		18/308 (5.8%)	93/308 (30.2%)	113/308 (36.7%)	224/924 (24.2%)
Confirmed <i>C. fetus</i> subsp. <i>venerealis</i>	Heifers	1/168 (0.6%)	66/168 (39.3%)	32/168 (19.0%)	99/504 (19.6%)
	Bulls	7/140 (5.0%)	14/140 (10.0%)	30/140 (21.4%)	51/420 (12.1%)
Media Totals		8/308 (2.6%)	80/308 (26.0%)	62/308 (20.1%)	150/924 (16.2%)

*Gram negative, microaerophilic, spiral motility, pink-grey shiny convex morphology

4.4 Discussion

The optimisation of culture media is usually undertaken using pure *C. fetus* subsp. *venerealis* cultures rather than spiked mixed cultures to simulate the isolation of *C. fetus* subsp. *venerealis* from clinical samples. These experiments have shown that a combination of plating with filtration and the use of selective agar is optimal for the isolation of *C. fetus* subsp. *venerealis* from mixed cultures using either TEM. While filtration of samples can produce lower numbers of *C. fetus* subsp. *venerealis* colonies, the technique excludes potential contaminants such as *P. aeruginosa*. Results also showed that direct plating onto SBA plates is only useful for pure *C. fetus* subsp. *venerealis* cultures, while selective agar is more useful in the presence of certain contaminating organisms. In addition, this study showed that using a TEM produce higher *C. fetus* subsp. *venerealis* growth than direct plating from PBS, with Lander's producing the highest rate of *C. fetus* subsp. *venerealis* isolations.

Direct plating of stored contaminated stocks of *C. fetus* subsp. *venerealis* onto SBA resulted in complete overgrowth. This may be due to contaminating bacteria overgrowing the fastidious *C. fetus* subsp. *venerealis* strains which are particularly slow growing when resuscitated from -80°C storage (results not shown). While enrichment may help increase the concentration of *C. fetus* subsp. *venerealis*, it may also increase of the concentration of non-*C. fetus* organisms and the technique was shown to be considerably less successful than direct filtration. Success in the recovery of stored *C. fetus* subsp. *venerealis* strains by direct filtration could be due to the known high concentration of bacterial cells stored down. This is imperative as filtration has been shown to greatly reduce the resultant number of *C. fetus* subsp. *venerealis* colonies (Lander 1990, Chaban, Guerra et al. 2013) as was evident using pure *C. fetus* subsp. *venerealis* cultures in Experiment 2. For the filtration of mixed *C. fetus* subsp. *venerealis* samples, a 0.45 µm filter was selected compared to 0.65 µm filters used in other studies to reduce the presence of smaller contaminants such as *P. vulgaris* (0.4 – 0.6 µm wide, 1.2 – 2.5 µm long), while still allowing the isolation of *C. fetus* subsp. *venerealis* (0.3 – 0.4 µm). *P. aeruginosa* (0.5 – 0.8 µm wide, 1.5 – 3.0 µm long), should theoretically be excluded using either a 0.45 or 0.65 µm filter. Direct filtration was also done without any incubation of the stored broth, therefore, not allowing the contaminating organisms to replicate and undoubtedly overgrow the much slower growing *C. fetus* subsp. *venerealis*. It is therefore noteworthy that filtration can be successfully employed on known positive media in the presence of contaminating organisms.

Filtration was however not successful from TEMs spiked with pure *C. fetus* subsp. *venerealis* mixed with *P. aeruginosa*. The concentration of *C. fetus* subsp. *venerealis* in these samples was lower than that stored down in broths used in Experiment 1 which may account for negative results in Experiment 2. The limited nutritional and growth support offered by TEMs may have been expended on *P. aeruginosa* within the incubation period, leaving no viable *C. fetus* subsp. *venerealis* colonies. It may also be indicative of too high a concentration of *P. aeruginosa* used in the experiment simulation or strain specificities making it highly competitive.

Filtration was successful in allowing isolation of *C. fetus* subsp. *venerealis* from *P. vulgaris* mixed Lander's when cultured within 48 h. However, positive culture results were obtained with Campylobacter agar plates after 48 h incubation only. Whether this was due to subsampling discrepancies, such as the depth of the TEM broth sampled for plating may need further research. Campylobacter agar plates were successful in partially hindering the growth of *P. vulgaris* regardless of TEM or incubation temperature, with at most, moderate growth of the organism on culture media. Other research has also shown that selective agar can be useful in allowing successful isolations from spiked mixed cultures with 46.5% of samples positive on Skirrow's agar, and only 28.6% positive on SBA in one study (Monke, Love et al. 2002). A second study found that 40% of positive isolations were made using Skirrow's agar compared to 35% on SBA after enrichment in TEM (Chaban, Guerra et al. 2013). This research has shown that Campylobacter agar, with variations in antibiotics included compared to the commonly used Skirrow's agar, is also able to allow successful isolations in specific mixed cultures. However, further research into the inhibition of a wider range and combination of contaminating organisms is required.

TTE and Lander's were equally able to allow isolations from *P. vulgaris* or *C. jejuni* mixed cultures for up to 7 days, with higher *C. fetus* subsp. *venerealis* colony counts seen on culture media inoculated from TTE. These findings were different to the results previously reported where isolation of *C. fetus* subsp. *venerealis* was possible after 8 days in TTE and only after 4 days in Lander's incubated at 37°C (Harwood, Thomann et al. 2009). This inconsistency may be due to the concentration inoculated, with 10³ CFU/ml compared to 10⁵ CFU/ml used in our study or due to strain differences. Strains have been shown to vary geographically by molecular testing (Wagenaar, van Bergen et al. 2001), and it is therefore feasible that strains as well as biovars may display biochemical differences or media preferences. The increased colony counts from TTE cultures may be indicative of TTE providing a better enrichment medium if samples require greater than 72 h transport, though further research is needed to support these findings, especially compared to a

range of different contaminants due to the reduced antibiotic concentrations in TTE. Harwood et al (2009) showed that the concentrations of antibiotics included in Lander's were inhibitory to the *C. fetus* subsp. *venerealis* strain tested in TTE, producing no growth at all (Harwood, Thomann et al. 2009). Reduced concentrations of the same antibiotics; vancomycin, trimethoprim and polymyxin B allowed a 3-log increase in concentration of the same *C. fetus* subsp. *venerealis* strain in TTE. The combination of antibiotics used in Lander's was developed by Skirrow almost 40 years ago and is still used in its same concentrations as a supplement available for commercial purchase. The only variation to this has been the reduction of polymyxin B by Hum due to findings of 90.0% of tested *C. fetus* subsp. *venerealis* being sensitive to this antibiotic (Jones, Davis et al. 1985). Further research is needed to determine the antimicrobial susceptibilities of a wide range of *C. fetus* subsp. *venerealis* biovars and strains, particularly for those included in TEMs as the Skirrow's supplement was initially developed for the isolation of *C. coli* and *C. jejuni* (Skirrow 1977).

Differential incubation of samples at 25°C supported the growth of *C. fetus* subsp. *venerealis* in the presence of *P. vulgaris* or *C. jejuni* with positive culture from Lander's and TTE after 24 and 48 h incubation respectively. This is important as it is recommended to culture from transport medium upon receipt (Monke, Love et al. 2002, OIE 2012, Chaban, Guerra et al. 2013) as opposed to 72 h incubation as found previously (Lander 1990, Hum, Hornitzky et al. 2009). It was found that incubation of a range of spiked media (Weybridge, Cary-Blair and 0.85% saline) overnight reduced the culture success rate by 17.3% in one study (Monke, Love et al. 2002), while a second study found direct plating compared to overnight incubation of samples in TEM reduced culture rates by up to 51% (Chaban, Guerra et al. 2013). An earlier study by Hum also found that direct culturing of bovine preputial and vaginal mucus samples produced the highest success rate with 94.7% of positive samples (n=19) detected by this method, and 73.7% detected after incubation in Lander's at 37°C for 72 h (Hum, Brunner et al. 1994). This study showed that incubation of TEMs at 25°C improved the isolation of *C. fetus* subsp. *venerealis* from mixed cultures. Further research using this reduced incubation temperature should be tested in spiked preputial samples and field trials before implementing in diagnostic laboratories.

Therefore, Experiment 2 identified that Lander's TEM and Campylobacter selective plates were able to produce higher *C. fetus* subsp. *venerealis* culture success rates from spiked mixed cultures. Size (filtration) and antimicrobial exclusion (selective agar) reduced the growth of *P. vulgaris* or *C. jejuni* in *C. fetus* subsp. *venerealis* mixed cultures, with the initial concentration of *C. fetus* subsp. *venerealis* in the sample defining filtration success. However, in this study, contamination with *P.*

aeruginosa while able to be excluded using filtration, was unable to produce a positive *C. fetus* subsp. *venerealis* result regardless of the TEM or culture media. This may be due to nutrient depletion caused by the *P. aeruginosa* or other inhibitory aspects. Further studies would need to elucidate the competitive mechanisms of *Pseudomonas* growth in comparison to *C. fetus* subsp. *venerealis* enrichment which impact on the survival of viable cells. TEMs appeared to be similarly efficacious with isolations from pure spiked samples or *P. vulgaris* or *C. jejuni* contaminated samples from both Lander's and TTE. However, isolations from samples contaminated with the *P. vulgaris* or *C. jejuni* were only possible from short incubations (≤ 48 h) at 25°C for *P. vulgaris* and predominantly from 25°C incubation compared to 37°C incubation with *C. jejuni*.

In the simulated *in vivo* collections from experimental infections of two strains of *C. fetus* subsp. *venerealis*, both TTE and Lander's TEMs were able to sustain and enrich both biovars of *C. fetus* subsp. *venerealis*. There was a noticeable, though not significant difference in the success rates of media specific isolations from heifers or bulls. Lander's TEM appeared to allow increased success of isolating *Campylobacter* from heifers while TTE produced higher isolations from bulls. While it is unknown what could account for the preferences for either media, it is speculated that the similar pH levels of vaginal mucus (7.3 – 7.6) (Beckwith-Cohen, Koren et al. 2012) and Lander's TEM (7.4 – 7.6) may better support bacteria from heifer samples. The pH of TTE is 6.8 - 7.1 which may be more conducive for culture from bull samples due to the pH of semen being 6.2 – 7.0 (Kirton, Hafs et al. 1964). Further research to confirm this observation in order to develop better cultures guidelines for different clinical samples is warranted.

A limitation of this study, as a culture assessment trial, was the unknown status of the animals tested. The study was an infection trial, and it was unknown how effective the two chosen strains were at establishing infection or clearing of infection during the sampling period. The infected status of the animals could be assumed due to positive culture results over the sampling period, however, transient infections could account for peak in isolations at week two in heifers and week five in bulls. Other biological factors could affect the culture results in heifers, such as changes in oestrus cycle, with 82% positive culture results detected from vaginal mucus samples collected at oestrus in one study compared to 28% at dioestrus (Laing 1956). However, other studies have found the opposite in bacteriological isolations of bovine uterus flora (Dawson 1959). The culture trend in our study more closely supported the second finding, with culture peak at week two which would have been at dioestrus, followed by a second peak in culture at week five. The high culture success early in the trial could also be due to the stage of infection, with studies mentioning that isolations

from heifers are more likely during the acute phase of infection, which is typically 10 – 30 days post-infection (Laing 1956). It is believed that clearing of the organism in heifers occurs between weeks 6 – 12 (Clark 1971, Bondurant 2005), which may be indicated by the decrease in culture success seen in our study at week 6. In bulls, the age of the animal could impact on the ability of the strains to produce sustained infection as it is speculated that older bulls with deeper preputial crypts may be more susceptible to persistent infections (Samuelson and Winter 1966) while younger bulls (< 4 years old) are generally only transiently infected (Wagner, Dunn et al. 1965, Dufty, Clark et al. 1975).

Samples were collected into a consistent volume across all media, in 9 ml culture tubes to ensure the Tricamper™ was completely submerged to allow maximum access to any scrapings along the length of the device, as well as reduce variations in concentrations of bacteria across the TEMs. The effects of temperature on viability of *C. fetus* subsp. *venerealis* may have affected the isolation results, as the sampling was conducted during the SE Qld summer with mean temperature for the UQ Pinjarra Hills Farm of 29.7°C in February and maximum temperatures reaching 34.7°C. Due to the large number of animals in our study, the time from collection to processing, including transport back to the laboratory (~25 km), often lasted approximately 5 - 6 h.

All cultures isolated from samples were morphologically, microscopically and biochemically profiled. A proportion of samples returned *Campylobacter*-like organisms that had a similar morphology, grew microaerobically and displayed the unique darting corkscrew motility. However, biochemical typing of these isolates found over half of the bull cultures (58.2%) to have varying biochemical results. These may have been *C. sputorum* isolates, which have been shown to be present in bull preputial samples (Samuelson and Winter 1966), but confirmatory testing was not conducted. Conversely, almost all heifer isolations (97.1%) were biochemically confirmed as *C. fetus* subsp. *venerealis*. The variation seen in the bull isolates compared to those from heifers could be due to a more robust bacterial flora in penile sheaths (Ruebke 1951) providing a variety of contending species for isolation, or biochemical testing inaccuracies. Further studies into bacterial strains present in Australian bull prepuce or heifer vaginal samples would be advantageous in ascertaining the normal flora and prevalence of non-pathogenic *Campylobacter* strains. In addition, due to the large influx of samples, different laboratory technicians assisted in the screening of the isolates and it is possible human error may have contributed to the variation in biochemical results. The most common test seen to be misinterpreted is the test for oxidase. Although batch quality control is checked weekly, staff unfamiliar with screening *C. fetus* subsp. *venerealis* may not have

used enough culture thus discarding the isolate as oxidase negative. H₂S testing of the reisolated inoculated strains from group 924 animals were often negative (61.5%) which could also be due to failings of the test methodology or unreliability.

A Canadian study into the success of culture from smegma scrapings from infected bulls found that the use of PBS with passive filtration through a 0.65 µm filter produced a high level of isolations (86.5%) with 37.5% of samples positive after incubation in Lander's TEM (Chaban, Guerra et al. 2013). This study however, used four naturally infected and eight artificially infected bulls, with no mention of the infection dose or ages of the animals. A higher concentration of bacteria could account for the differences seen in isolation success using a similar culture methodology as well as differences in media volumes. The same study used 2 ml of PBS for collection of samples of which 300 µl was transferred into 10 ml of TEM, potentially diluting out the bacteria (Chaban, Guerra et al. 2013). Comparison of saline (0.85%) and Lander's in a laboratory trial using spiked bull preputial samples found 18.1% and 67.2% of positive isolations, respectively (Monke, Love et al. 2002). These results are similar to the variation seen in our research where PBS offered the lowest level of isolation compared to enrichment in TEMs. This may be suggestive of the effect of enrichment offered by TEMs compared to PBS/saline even over a short period of time allowing improved growth and viability of *C. fetus* subsp. *venerealis*.

Other *C. fetus* subsp. *venerealis* isolation studies undertaken in the USA and Canada (Monke, Love et al. 2002, Chaban, Guerra et al. 2013) do not mention the presence of other *Campylobacter* species during isolation, which may be suggestive of this being a problem in tropical countries such as Australia. Whether these thermophilic *Campylobacters* are more apparent in samples collected from tropical and sub-tropical climates of Australia requires further investigation and how this may affect isolation and identification protocols which currently do not emphasize differentiation of *Campylobacter* species. A higher level of isolations of other *Campylobacter* species was seen in this and related student studies in Australia (Indjein 2013, Nordin 2013) which is indicative of further research required into differentiating between the species, and producing enrichment and isolation methodologies that favour *C. fetus* subsp. *venerealis*.

Overall, this study has shown that the TEMs, Lander's and TTE, are able to sustain and enrich *C. fetus* subsp. *venerealis*, in simulated *in vitro* and *in vivo* field trials. The inclusion of selective agar and filtration methods in routine culture techniques may prove useful for the isolation of *C. fetus* subsp. *venerealis* in the presence of mixed cultures (*P. aeruginosa*, *P. vulgaris* and *C. jejuni*).

Direct plating onto non-selective agar produced the least number of positive *C. fetus* subsp. *venerealis* cultures in the presence of these spiked bacteria. While filtration was useful in this study, a higher concentration of *C. fetus* subsp. *venerealis* is needed thus parallel plate with no filter is needed to isolate from samples with lower concentrations of *C. fetus* subsp. *venerealis*. Incubation at 25°C (instead of 37°C) was shown to favour *C. fetus* subsp. *venerealis* growth in the presence of *P. vulgaris* and *C. jejuni* which may not always be excluded by filtration, selective media or antibiotics. If these laboratory findings, using spiked mixed cultures could be extrapolated to the majority of contaminating organisms found in submitted samples, it may provide an easily implemented method to improve *C. fetus* subsp. *venerealis* isolations and diagnostic success. Further research is needed into pH or antimicrobial specificities to allow for further optimization of TEMs, specifically for type or gender origin of sample, as well as to allow isolation of *C. fetus* subsp. *venerealis* strains present in a sample regardless of its antibiotic susceptibilities.

Chapter 5. Biochemical
Characterisation: A comparison of
culture phenotyping with automated
technologies

5.1 Introduction

Identification of *C. fetus* subsp. *venerealis* is extremely important given BGC trade restrictions as well as incidences of infertility and sporadic abortions in herds. Once a suspect colony has been isolated from a submitted sample, biochemical characterisation of the sample needs to be accurately conducted to allow diagnosis. While there are standard tests for the differentiation of colonies, as outlined in the OIE and national protocols, different applications of these methodologies can lead to discrepant results.

Distinction between *Campylobacter* species and subspecies tends to rely on single biochemical tests, which highlights the importance of every test result in the context of diagnoses. Glycine tolerance, can be used to distinguish between *C. fetus* subsp. *fetus*, a more common ovine pathogen which is responsible for sporadic abortions in cattle, and *C. fetus* subsp. *venerealis* which can cause abortion and infertility in cattle at outbreak proportions. However, the reliability of this test has been questioned due to mediation by phages of the genes responsible for glycine intolerance (Chang and Ogg 1971) and further phenotypic markers for distinction between the subspecies are sought. Studies have shown variable correlation with biochemical testing and molecular identification with one study identifying conformity between glycine tolerance and molecular results of 103 tested strains using the duplex PCR developed by Hum (Schulze, Bagon et al. 2006). However, a second study found that biochemical characterisation using glycine tolerance was responsible for three misidentifications of strains (n=33) as *C. fetus* subsp. *fetus* which were actually *C. fetus* subsp. *venerealis* according to restriction fragment length polymorphism (RFLP) analysis (Vargas, Costa et al. 2003). Conversely, 7 strains biochemically identified as *C. fetus* subsp. *venerealis* with inability to tolerate glycine in one study were shown to be *C. fetus* subsp. *fetus* according to both the Hum duplex PCR and amplified fragment length polymorphism (AFLP) typing (Wagenaar, van Bergen et al. 2001).

Other commonly used distinguishing tests have been identified as variable or subjective, such as the distinction between the two biovars of *C. fetus* subsp. *venerealis*; Venerealis and Intermedius, according to the production of H₂S in a 0.02% cysteine broth, resulting in the darkening of lead acetate paper (Veron and Chatelain 1973). *C. fetus* subsp. *venerealis* bv. Intermedius isolates were positive on this test and was thus named 'Intermedius' as *C. fetus* subsp. *fetus* is also positive whereas bv. Venerealis is negative (Veron and Chatelain 1973) (hence 'intermediate' between the 2 subspecies). This test is highly subjective due to the variation in the darkening of the lead acetate strip detected visually. Growth at 42°C has also been shown to produce variable results for *C. fetus*

subsp. *venerealis*, with 9.9% of strains in a study tolerating the higher temperature (Schulze, Bagon et al. 2006). Though OIE suggest variable results for catalase, growth at 25°C and susceptibility to nalidixic acid (OIE 2012), the level of possible variations in *C. fetus* subsp. *venerealis* strain characteristics is unknown.

Some biochemical tests, such as 1% glycine can be impacted by inoculum size with 10⁶ CFU/ml required to produce reliable, reproducible results, while higher concentrations may produce false positives (On and Holmes 1991). Differences in laboratory specific implementations of common biochemical tests can lead to incorrect results, as was seen in a study where 99 *C. fetus* strains collected and profiled over a 10-year period were resuscitated from storage and phenotypically and molecularly characterised (Hum, Quinn et al. 1997). Of these, 10 isolates were shown to be *C. hyointestinalis* (n=8), one *C. jejuni*, and one *C. sputorum*. Similar results were found with our research as demonstrated in an Honours project (Nordin 2013). While variation in test results is apparent with *C. fetus* subsp. *venerealis* strains, an understanding of the probability that could be expected would allow prioritisation of the most reliable tests as well as defining where discrepancies are most likely to occur.

Furthermore, the presence of *Arcobacter* species in cattle digestive tracts and isolated from aborted bovine foetuses as a potential pathogen needs to be identified or excluded during diagnosis (Ho, Lipman et al. 2006). The most commonly isolated *Arcobacter* species in cattle is *Arcobacter cryaerophilus* (formerly *Campylobacter cryaerophilus*) which is distinguished from *C. fetus* subsp. *venerealis* by its aerotolerance, resistance to cephalothin and tolerance of lower temperatures (growth at 15°C) (Vandamme, Falsen et al. 1991). *Arcobacter cryaerophilus* has been found in faecal samples from healthy cattle across three different farms in one study, with 26% of samples showing co-colonization with other *Arcobacter* species (Driessche, Houf et al. 2005). Limited information is available on the pathogenicity or biochemical characterisations of even the more common *Arcobacter* species, and continuing research is needed to allow better identification and diagnosis of this organism.

Implementation of automated testing systems as is used in most medical diagnostic laboratories for a variety of other common bacterial species such as *E. coli*, *staphylococcus* and *streptococcus* would reduce laboratory or technician specific error as well as produce a user-friendly, and more reproducible set of results. Analytical profile index (API) kits and Biolog tests have not been used for the identification of *C. fetus* subsp. *venerealis* which is likely due to the disparities seen in

culture phenotyping. API Campy kits reportedly identified 14 *Campylobacter* species, with variable results during implementation (Huysmans, Turnidge et al. 1995, Reina, Ros et al. 1995, Martiny, Dediste et al. 2011). A study has shown that API Campy kits have a high level of accuracy for the identification of medical *C. jejuni* isolates, with complete conformity between API and culture phenotyping (oxidase, catalase, growth at 42°C, sensitivity to nalidixic acid and cephalothin and hippurate hydrolysis) (Huysmans, Turnidge et al. 1995), and 94.4% agreement in a second study (Martiny, Dediste et al. 2011). A lower level of identification was seen with medical *C. coli* and *C. lari* isolates with 26 – 75% accuracy with the former and 0 – 33% with the latter across different studies (Huysmans, Turnidge et al. 1995, Reina, Ros et al. 1995, Martiny, Dediste et al. 2011). The API Campy kit has not been used for diagnosis of *C. fetus* strains of veterinary origin, although the species are included in the API results online database (Biomérieux 2015). If research showed a higher or equal level of correlation with API kit testing than is possible with culture phenotyping, it could provide a more user-friendly and cost-effective identification method for *C. fetus* isolates in diagnostic laboratories.

Similarly, Biolog has been used as a metabolic characterisation method for a range of bacterial species of medical and veterinary significance, and while it has been used for *C. jejuni* and *C. coli* (Line, Hiatt et al. 2010, Line, Seal et al. 2011), no research has explored the validity of the method in defining a profile for *C. fetus* subsp. *venerealis*. Temperature associated virulence factors were identified in these *Campylobacter* species, and similar research could be of interest within *C. fetus* as limited knowledge is available on its virulence factors and mechanisms. With the availability of 1,920 assays over the test Phenotyping MicroArray plates (Bochner 2009), there is the potential to recognise a large number of substrates which may provide differentiating tests for *Arcobacter* and *Campylobacter* species from *C. fetus* subspecies and biovars, or for implementation of this knowledge into the improvement of specific transport and growth media. The Biolog microplates allow analysis of the organism's metabolic processes and cellular pathways by measuring the respiration levels of the inoculated bacteria in response to the alternative sources of different assay products (Bochner 2009). The Biolog GEN III system (Biolog, Hayward, CA), with a single plate consisting of 94 test substrates is able to provide a quicker bacterial identification system than the 20 plate Phenotyping MicroArray (Biolog, Hayward, CA) and may be implemented as a more routinely used test if a *C. fetus* subsp. *venerealis* profile could be developed.

Therefore this chapter aims to biochemically characterise 152 available *Campylobacter* isolates using OIE recommended culture phenotyping tests as well as subsets of isolates comparative to 1)

API Campy kits, 2) Biolog Phenotypic MicroArray and 3) Biolog GEN III systems. This chapter also aims to provide an indication of the antibiotic sensitivities of seven *Campylobacter* strains. The aim is to provide an overview of the level of variation seen in Australian *C. fetus* subsp. *venerealis* strains as well as the reliability of currently used golden standard assays for differentiating species such as glycine tolerance and temperature or aerotolerance.

5.2 Materials

5.2.1 Media

All media used in this chapter for the resuscitation, growth or profiling of isolates has been included in Chapter 3. Reagents for API Campy kits and Biolog were obtained from the respective manufacturers, BioMérieux (bioMérieux, Marcy-l'Etoile, France) and Biolog (Biolog, Hayward, CA).

5.2.2 Isolates*

A total of 152 *Campylobacter* isolates were profiled. These include 1) isolates collected during a previous candidature study (Indjein 2013) from an abattoir bull prepuce survey (n=54), 2) Biosecurity Queensland laboratory (Coopers Plains, Brisbane, Queensland, Australia) type strains for *C. fetus* subsp. *venerealis* (Q41 = ATCC19438) and *C. jejuni* (Q6 = ATCC33291) (n=2), 3) *C. fetus* subsp. *venerealis* isolates from diagnostic sample submissions to the Biosecurity Queensland laboratory (n=14), 4) *Campylobacter* isolations during this candidature from herd screening across Queensland, Australia (n=74), 5) 2 reference *C. fetus* subsp. *venerealis* biovar strains; bv. *Venerealis* strain (B6) and bv. *Intermedius* (642-21) (Donaldson and Clark 1970), 6) two *C. fetus* subsp. *fetus* (BT54/00; BT277/06) and two *C. fetus* subsp. *venerealis* (BT08/04; BT102/00) strains shared from a diagnostic laboratory in the United Kingdom (Veterinary Laboratories Agency (Weybridge), New Haw, UK) (n=4), 7) ATCC type strains; ATCC 19438 – *Campylobacter fetus* subsp. *venerealis*; and ATCC 25936 – *Campylobacter fetus* subsp. *fetus* (n=2) (Table 17).

*The terminology 'isolates' refers to bacterial strains that have been isolated but not yet identified, while 'strains' refers to those that have been characterised as a particular species.

5.3 Methods

5.3.1 Culture Phenotyping

All isolates (n=152) were resuscitated or isolated as previously mentioned (Chapter 4). Isolates were checked for colony conformation, Gram stain (negative) and corkscrew motility. Strains were then profiled using OIE standardised differentiating tests: oxidase, catalase, growth at 25°C and 42°C, H₂S production on a triple sugar iron (TSI) slope and in 0.02% cysteine broth, growth in 1% glycine and antimicrobial sensitivity to nalidixic acid, and cephalothin (see Table 4) (OIE 2012) and additionally growth aerobically and antimicrobial sensitivity to tetracycline and streptomycin. Tests were only carried out on pure cultures with aerobic and microaerobic purity controls, using standard inocula for each test; single isolated colonies for catalase and oxidase, 100 µl of a 10⁷ CFU/ml inoculum for TSI slope, cysteine broth and glycine broth inoculation and lawns on SBA plates were streaked using a 0.5 McFarland standard concentration in saline for antibiotic disk testing (CLSI 2012).

5.3.2 Antibiotic Profiling

A subset of 6 strains which were of interest for animal model use (Chapter 4 - Experiment 3, Chapter 6 and 7) were selected for profiling using 27 different antibiotics disks; one *C. fetus* subsp. *fetus* (ATCC25936), four *C. fetus* subsp. *venerealis* (B6, 642-21, 540 and 635) and one *C. hyointestinalis* (830). Strains were resuscitated and passaged twice to ensure purity. Plates incubated for 48 h were used to produce a lawn by selecting isolated colonies with a sterile swab which were washed off into saline to produce a 0.5 McFarland standard turbidity (CLSI 2012). This was used to streak 9 SBA plates in three directions to produce a lawn. Plates were allowed to dry before three antibiotic disks were placed on each one, and incubated in the microaerobic workstation for 72 h. Plates were read using calibrated callipers and zones of inhibition recorded for each disk. A zone of 6 mm was interpreted as no inhibition and is equivalent to the disk diameter (CLSI 2012). *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923 were used as quality control species for antimicrobial disk performance and repeatability according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012).

5.3.3 API Campy kit

API Campy kits (BioMérieux) were used to profile a subset of strains consisting of; 23 from the abattoir preputial survey (154, 530, 531, 534, 537, 540, 635, 672, 673, 676, 729, 742, 744, 830, 849, 863, 882, 923, 924, 926, 951, 957, 979), four UK isolates (BT54/00, BT277/06, BT08/04, BT102/00), one ATCC reference strain (ATCC19438), four Biosecurity Queensland isolates (Q41, M08/16749, M09/10806 and 101302) two biovar reference strains (B6 and 642-21) and eight isolated in this candidature (PhD 2-64, 66, 67, 68, 70, 72, 73) (n=42). Isolates were resuscitated and passaged at least twice to ensure purity. The kits were used according to the manufacturer's instructions. The inoculated strips were incubated for 48 - 72 h in the microaerobic chamber (Don Whitley Scientific Pty Ltd, North Gosford, NSW, Australia) on distilled water moistened paper towel to prevent desiccation of substrates. The test strips were checked at 48 h for positive sodium succinate test. If positive, strips were read according to the manufacturer's instructions and the acquired numerical profile input into the API identification website (Biomérieux 2015) to produce a result. If the sodium succinate test was negative, the strips were incubated for a further 24 h before being read.

5.3.4 Biolog GEN III™

A further subset of strains (n=27) of interest were used to assess the Biolog GEN III assay including the two ATCC strains (ATCC19438 and ATCC25936), two Biosecurity Queensland laboratory reference strains (*C. fetus* subsp. *venerealis* (Q41) and *C. jejuni* (Q6)), two biovar reference strains (B6 and 642-21), four Biosecurity Queensland laboratory diagnostic strains (75838, 76223, M08/16749 and M09/10806), two isolated in this candidature (39-8, A8) and 15 isolated in a previous candidature from the abattoir prepuce survey (527, 534, 540, 543, 635, 676, 742, 744, 830, 849, 863, 924, 926, 957, 979) (Indjein 2013). Biolog GEN III (Biolog, Hayward, CA) is a single 96-well microplate consisting of 94 biochemical tests; 71 carbon sources and 23 chemical sensitivity assays. All plates were inoculated according to protocol C2 – fastidious, oxygen sensitive, Gram negative organisms. Resuscitated isolates were passaged twice to ensure purity. Multiple SBA plates were then streaked and incubated for 48 h to provide adequate cell density. The culture from these plates was harvested using a sterile swab, inoculated into inoculating fluid IF-C, and the turbidity checked to be in the range of 62 – 68% transmittance using a turbidimeter (Biolog, Hayward, CA). Once the desired turbidity was reached, the plate wells were inoculated with 100 µl using a multichannel pipette aseptically and the plate was immediately sealed. Two purity plates were streaked from the remaining inoculum and incubated aerobically and microaerobically to check sterility of inoculating fluid. Microplates were incubated in the microaerobic workstation on

distilled water moistened paper towel to prevent wells dehydrating, for 48 – 72 h. Microplates were read using Biolog's microbial identification system with Omnilog software (Biolog, Hayward, CA).

5.3.5 Biolog Phenotypic MicroArray™

A total of 10 strains were tested; four *C. fetus* subsp. *venerealis* strains in duplicate (ATCC19438, 635, B6 and 642-21) and a further 6 strains (*C. fetus* subsp. *fetus* ATCC25936; *C. fetus* subsp. *venerealis* A8, 540, 830, 924 and 957) singularly. The Biolog Phenotype Microarray (PM) (Biolog, Hayward, CA) consists of 20 microplates comprising 1,920 assays that physiologically define bacteria using carbon, nitrogen and biosynthetic pathways. Due to budget constraints, 13 plates were selected for use in this study; two carbon source plates, one pH gradient plate and 10 chemical sensitivity substrate plates. The manufacturers manual stipulates that strains resuscitated from frozen or lyophilized storage should be subcultured a minimum of three times prior to use, and strains should be tested prior to reaching stationary phase of growth. Therefore, isolates were resuscitated and grown as per section 3.3, subcultured three times and used at 48 h growth. The inoculum was prepared as per manufacturer's instructions Protocol C for *Campylobacter jejuni* (Biolog 2012) with incubation in the microaerobic chamber for 48 – 72 h on distilled water moistened paper towel to prevent desiccation. Plates were read in a Multiskan Ascent plate reader (Thermo Labsystems, ThermoFisher Scientific, Adelaide, SA, Australia). For strains tested in duplicate, the second test was carried out at a different time point using the same method described above. Plates were analysed by a biometrician using statistical software (CoPlot, CoHort Software, Monterey, CA) instead of the Omnilog analysis program (Biolog, Hayward, CA) due to unaffordability of the software. Absorbance levels were calculated by subtracting the negative well value from the readings of all other wells to obtain the adjusted absorbance value which was plotted for each well across each plate.

5.4 Results

5.4.1 Biochemical Tests

A total of 44 isolates (28.9%) (n=152) matched the OIE profile for *C. fetus* subsp. *venerealis* identification for both biovars Intermedius and Venerealis. The number of isolates showing differences in the characteristics previously identified to show variable results; catalase, growth at 25°C and resistance to nalidixic acid, were between 2 - 8 isolates per test, though overall, a total of 10 strains produced variable results on a combination of the tests (Table 10). The two known *C. fetus* subsp. *fetus* strains shared from the UK laboratory (BT54/00; BT277/06) both returned negative glycine results, identifying them as *C. fetus* subsp. *venerealis*. A further 54 isolates (35.5%) (n=152) were identified as either *C. fetus* subsp. *fetus* (n=2), *C. jejuni* (n=17), *C. hyointestinalis* (n=13), *C. sputorum* (n=18) or *Arcobacter cryaerophilus* (n=4) (Table 11). Of these isolates identified as other *Campylobacter* species (n=54), 36 isolates (66.7%) were characterised by thermophilic tolerance (growth at 42°C). A total of 65 isolates (42.8%) were unidentified by the differentiating biochemical tests included in the profile. Of these unidentified isolates (n=65), 37 (56.9%) were negative on oxidase testing. There were 6 isolates within these which fit the biochemical profile of *C. fetus* subsp. *venerealis*, except for their resistance to cephalothin (Table 12). All of the biochemically confirmed *C. fetus* subsp. *venerealis* biovar Venerealis isolates displayed sensitivity to tetracycline and streptomycin and two *C. fetus* subsp. *venerealis* biovar Intermedius strains displayed resistance to streptomycin with one of these also resistant to tetracycline (Table 12). It was noted that morphologically, thermophilic *Campylobacter* species appeared slightly different to *C. fetus* strains with a slight green tinge to colonies and with a faster growth rate (results not shown).

Table 10. Biochemical analysis of 44 isolates identified as *C. fetus* subsp. *venerealis* including biovar differentiation and variable results for catalase, 25 °C growth and nalidixic acid sensitivity testing as recommended by the OIE (OIE 2012).

OX	CAT	25°C	42°C	TSI	H ₂ S	GLY	O ₂	NA	K	No. strains	Biochemical Identification
+	+	+	-	-	-	-	-	R	S	19	<i>C. fetus</i> subsp. <i>venerealis</i> biovar Venerealis
	-									2	
		-								8	
								S		4	
+	V	V	-	-	-	-	-	V	S	30	
+	V	V	-	-	+	-	-	V	S	15	<i>C. fetus</i> subsp. <i>venerealis</i> biovar Intermedius
Total										44	<i>C. fetus</i> subsp. <i>venerealis</i>

OX – oxidase; CAT – catalase; TSI – hydrogen sulphide production on a triple sugar iron slope; H₂S – hydrogen sulphide production in a 0.02% cysteine broth; O₂ – aerobic growth; NA – sensitivity to nalidixic acid; K – sensitivity to cephalothin; + positive result; - negative result; V – variable results; R – resistant; S - sensitive

Table 11. Biochemical analysis of 54 isolates identified as one of five other commonly occurring *Campylobacter* species in bull preputial or heifer vaginal mucus samples, according to OIE classification (OIE 2012)

Biochemical Identification	OX	CAT	25°C	42°C	TSI	H ₂ S	GLY	O ₂	NA	K	No. isolates
<i>C. fetus</i> subsp. <i>fetus</i>	+	+	+	V	-	+	+	-	R	S	2
<i>C. jejuni</i> ^a	+	V	-	V	-	N/A	V	-	V	N/A	17
<i>C. hyointestinalis</i>	+	+	V	+	V	N/A	V	-	R	N/A	13
<i>C. sputorum</i>	+	V	-	+	+	N/A	+	-	V	N/A	18
<i>Arcobacter cryaerophilus</i>	+	+	+	-	-	-	V	+	S	V	4 ^b
Total											55

OX – oxidase; CAT – catalase; TSI – hydrogen sulphide production on a triple sugar iron slope; H₂S – hydrogen sulphide production in a 0.02% cysteine broth; O₂ – aerobic growth; NA – sensitivity to nalidixic acid; K – sensitivity to cephalothin; + positive result; - negative result; V – variable results; R – resistant; S – sensitive; N/A – information not available; a - large overlap with *C. fetus* subsp. *venerealis* and *C. hyointestinalis* strains due to variable nature of results; b - according to (Schroeder-Tucker, Wesley et al. 1996)

Table 12. Antibiotic sensitivities of 44 biochemically identified *C. fetus* subsp. *venerealis* isolates (both biovars) to nalidixic acid (30 µg), cephalothin (30 µg), tetracycline (30 µg) and streptomycin (10 µg). Zone diameters for sensitivity as stipulated in OIE guidelines are 3 mm around disk or zone of 12 mm total (OIE 2012).

Number of strains	Nalidixic acid	Cephalothin	Tetracycline	Streptomycin
Resistant	36	6 ^a	1	2
Sensitive	8	44	43	42

R – resistant, S- sensitive; a - isolates not included in initial identification of *C. fetus* subsp. *venerealis* strains

Antibiotic profiling of the 6 selected strains which were of interest for further animal model virulence profiling due to preliminary antimicrobial results of interest or with virulence profiles matching that of vaccine strains showed that *C. fetus* subsp. *fetus* strains displayed greater resistance to six different antibiotics; ampicillin, cefuroxime, ceftiofur, cephalothin, novobiocin and penicillin, compared to tested *C. fetus* subsp. *venerealis* strains. *C. hyointestinalis* was also distinguished from *C. fetus* subsp. *venerealis* by smaller zones of inhibition with eight antibiotics; ampicillin, amoxicillin-clavulanic acid, cefuroxime, ceftiofur, enrofloxacin, novobiocin, penicillin and tetracycline (Table 12).

Table 13. Zones of inhibition (mm) using 27 different antimicrobial disks according to CLSI guidelines against one *C. fetus* subsp. *fetus*, four *C. fetus* subsp. *venerealis* and one *C. hyointestinalis* strains to identify differentiating zones in antibiotic sensitivities of species. (6.0 mm = no inhibition / disk diameter); dark grey shading - differentiating sensitivities between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* where there is alignment within subspecies results; light grey shading - differentiating sensitivities between *C. hyointestinalis* strain and aligned *C. fetus* subsp. *venerealis* strains results.

Strains Sequenced ID		<i>C. fetus</i> subsp. <i>fetus</i>	<i>C. fetus</i> subsp. <i>venerealis</i>				<i>C. hyointestinalis</i>
Antibiotic Disk	Disk Code	ATCC25936	B6	642-21	540	635	830
Ampicillin	AMP10	12.1	44.4	48.9	45.5	42.3	26.2
Amox-Clavul	AMC30	48.5	56.0	65.0	56.0	62.0	45.0
Apramycin	APR15	26.9	28.0	33.0	30.8	27.1	24.1
Bacitracin	BO.05	6.0	6.0	6.0	6.0	6.0	6.0
Cefuroxime	CXM30	18.2	48.0	62.4	64.0	51.0	34.4
Ceftiofur	EFT30	25.2	38.0	56.8	56.4	43.7	28.9
Cephalothin	KF30	25.1	50.6	52.2	54.0	51.2	19.7
Co-Trimoxazole	SXT25	6.0	6.0	6.0	6.0	6.0	6.0
Enrofloxacin	ENR5	39.8	43.0	49.4	48.0	48.8	33.9
Erythromycin	E15	39.5	31.9	30.5	34.5	32.7	30.6
Florenicol	FFC30	45.8	51.0	49.7	50.6	44.5	41.9
Furazolidone	FR100	46.4	54.0	57.0	42.6	55.6	47.8
Gentamicin	CN10	34.2	34.5	33.1	36.4	35.0	31.1
Lincospectin	LS109	40.5	36.4	44.0	37.0	35.3	39.4
Nalidixic acid	NA30	6.0	16.9	6.0	19.7	6.0	6.0
Neomycin	N30	35.7	35.9	42.8	34.4	33.9	34.1
Novobiocin	NV30	6.0	14.3	20.7	13.1	14.2	6.0
Oxacillin	OX1	6.0	6.0	6.0	6.0	6.0	6.0
Oxolinic acid	OA2	6.0	12.0	8.6	18.0	6.0	6.0
Penicillin	P10	22.5	50.4	52.7	66.0	49.0	32.9
Polymyxin B	PB300	19.7	22.1	21.9	23.3	21.3	21.1
Streptomycin	S10	31.1	34.4	35.0	36.0	31.3	32.0
Sulphonamides	S3300	6.0	6.0	6.0	6.0	6.0	6.0
Tetracycline	TE30	45.7	45.9	55.8	57.2	63.0	37.9
Ticarcillin	TIC75	44.7	66.0	62.8	65.6	53.0	54.6
Tilmicosin	TIL15	24.1	27.8	31.0	29.8	29.2	26.7
Trimethoprim	W5	6.0	6.0	6.0	6.0	6.0	6.0

5.4.2 API Campy kit

Of 20 strains biochemically characterised as *C. fetus* subsp. *venerealis*, 50.0% (n=10) correlated with API Campy kit results. This was lower than that of *C. sputorum* (64.3%; n=9) and *Arcobacter cryaerophilus* (100.0%; n=3) agreement. There was no agreement between *C. hyointestinalis* species biochemical identifications and API Campy kit identification (Table 5).

Table 14. Agreement between 42 species biochemical identity (as profiled in Tables 1 and 2) and API Campy kit identification of strains.

No of strains	Biochemical Identification	API Campy strain ID agreement
20	<i>C. fetus</i> subsp. <i>venerealis</i>	50.0%
5	<i>C. hyointestinalis</i>	0.0%
14	<i>C. sputorum</i>	64.3%
3	<i>Arcobacter cryaerophilus</i>	100.0%

5.4.3 Biolog GEN III Microplate

C. fetus strains did not consistently metabolise in the presence of 1% sodium chloride, while other *Campylobacter* species showed no metabolism with 1% sodium chloride, apart from isolate 830 (*C. hyointestinalis*). Isolate 543 was unable to produce an identification result based on biochemical testing yet was the only strain shown to be metabolically active at 4% sodium chloride, with strain 979 (*C. hyointestinalis*) the only strain active in 8% sodium chloride. All species variably tolerated pH 6, while none were metabolically active at pH 5. All negative control wells showed no catabolism of the substrate with positive results in all positive control wells. Only 10 of the 71 tested carbon sources were not tolerated for all isolates across all species; L-aspartic acid, D-serine, L-alanine, L-pyroglutamic acid, pectin, D-galacturonic acid, L-galacturonic acid lactone, p-hydroxyphenylacetic acid, γ -amino-butyric acid and sodium bromate. Further, methyl pyruvate catabolism appeared to have some level of agreement (79.3%; n=23) with H₂S production independently tested using lead acetate paper in a 0.02% cysteine broth. However, two *C. fetus* subsp. *venerealis* strains biochemically negative for H₂S production were positive for methyl pyruvate catabolism (Table 15).

Antibiotic catabolism showed variable results for rifamycin and lincomycin across tested strains and species (Table 16). Vancomycin showed respiration in all *C. fetus* subsp. *venerealis* tested, three *C. hyointestinalis* and the *C. jejuni* strain while all *C. sputorum* strains showed no respiration. There

was 62.1% (n=18) correlation between nalidixic acid substrate catabolism and the disk diffusion method for tested strains across all species.

Table 15. *Campylobacter* species respiratory metabolism of 1% sodium chloride, at pH 6 and of methyl pyruvate on Biolog Gen III microplate. The agreement of Biolog Gen III methyl pyruvate result to independent analysis of hydrogen sulphide (H₂S) production in a cysteine broth is also shown (grey highlighted). H₂S results shown to be variable (V) over multiple tests are interpreted as positive.

Isolate	Identification*	1% NaCl	pH 6	Methyl Pyruvate	H ₂ S*
ATCC19438	<i>C. fetus</i> subsp. <i>venerealis</i>	+	-	-	-
A8	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	+	-
M08	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	-	-
M09	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	-	-
Q41	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	-	-
B6	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	-	-
642-21	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	+	+
540	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	+	V
635	<i>C. fetus</i> subsp. <i>venerealis</i>	+	-	-	-
75838	<i>C. fetus</i> subsp. <i>venerealis</i>	-	+	+	+
76223	<i>C. fetus</i> subsp. <i>venerealis</i>	-	+	+	V
924	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	+	V
926	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	+	+
957	<i>C. fetus</i> subsp. <i>venerealis</i>	+	+	+	-
39-8	<i>C. fetus</i> subsp. <i>venerealis</i>	-	+	+	V
ATCC25936	<i>C. fetus</i> subsp. <i>fetus</i>	+	+	+	+
Q6	<i>C. jejuni</i>	-	+	-	-
676	<i>C. hyointestinalis</i>	-	+	-	+
742	<i>C. hyointestinalis</i> [^]	-	+	-	-
744	<i>C. hyointestinalis</i>	-	-	-	-
830	<i>C. hyointestinalis</i>	+	+	-	-
979	<i>C. hyointestinalis</i>	-	-	-	+
534	<i>C. sputorum</i>	-	-	-	+
849	<i>C. sputorum</i> [^]	-	-	-	+
863	<i>C. sputorum</i> [^]	-	-	+	+
527	N/A	+	+	-	-
543	N/A	+	+	-	-

*culture phenotyping; N/A – biochemical/ MLST identification not possible; + growth/ respiration; - no growth/ respiration; V – variable results; ^ MLST identification

Table 16. Response of tested *Campylobacter* strains (n=27) to antibiotic substrates (rifamycin, vancomycin, lincomycin and nalidixic acid) on Gen III microplate as well as comparison with nalidixic acid disk diffusion sensitivities. Strains with agreement between GEN III and nalidixic acid test results are highlighted grey.

Isolate	Identification*	Rifamycin	Vancomycin	Lincomycin	Nalidixic Acid	
					GEN III	Disk*
ATCC19438	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	R
A8	<i>C. fetus</i> subsp. <i>venerealis</i>	S	R	S	R	R
M08	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	S
M09	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	S
Q41	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	R
B6	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	S
642-21	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	R
540	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	S	S
635	<i>C. fetus</i> subsp. <i>venerealis</i>	S	R	S	R	R
75838	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	S	S
76223	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	S	S
924	<i>C. fetus</i> subsp. <i>venerealis</i>	S	R	S	R	R
926	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	S
957	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	R
39-8	<i>C. fetus</i> subsp. <i>venerealis</i>	R	R	S	R	R
ATCC25936	<i>C. fetus</i> subsp. <i>fetus</i>	R	S	R	R	R
Q6	<i>C. jejuni</i>	R	R	S	S	N/A
676	<i>C. hyointestinalis</i>	R	R	S	R	R
742	<i>C. hyointestinalis</i> [^]	S	S	S	S	R
744	<i>C. hyointestinalis</i>	R	R	S	R	R
830	<i>C. hyointestinalis</i>	S	S	S	R	R
979	<i>C. hyointestinalis</i>	S	R	R	R	R
534	<i>C. sputorum</i>	S	S	S	S	R
849	<i>C. sputorum</i> [^]	S	S	S	S	R
863	<i>C. sputorum</i> [^]	S	S	S	S	R
527	N/A	S	S	S	R	S
543	N/A	R	S	R	R	N/A

*culture phenotyping; N/A – biochemical/ MLST identification not possible; S = sensitive/ no respiration; R = resistant/ respiration;

[^] MLST identification

5.4.4 Biolog Phenotype MicroArray™

There was variable reproducibility across the four duplicate tested strains (ATCC19438, 635, B6 and 642-21) over the 13 plates, with plates 1 and 2 (carbohydrate sources) across all four strains showing a much higher level of reproducibility compared to plates 10 to 20 (pH and chemical sensitivity sources) (Figure 1). Substrate identities are shown in Appendix 1.

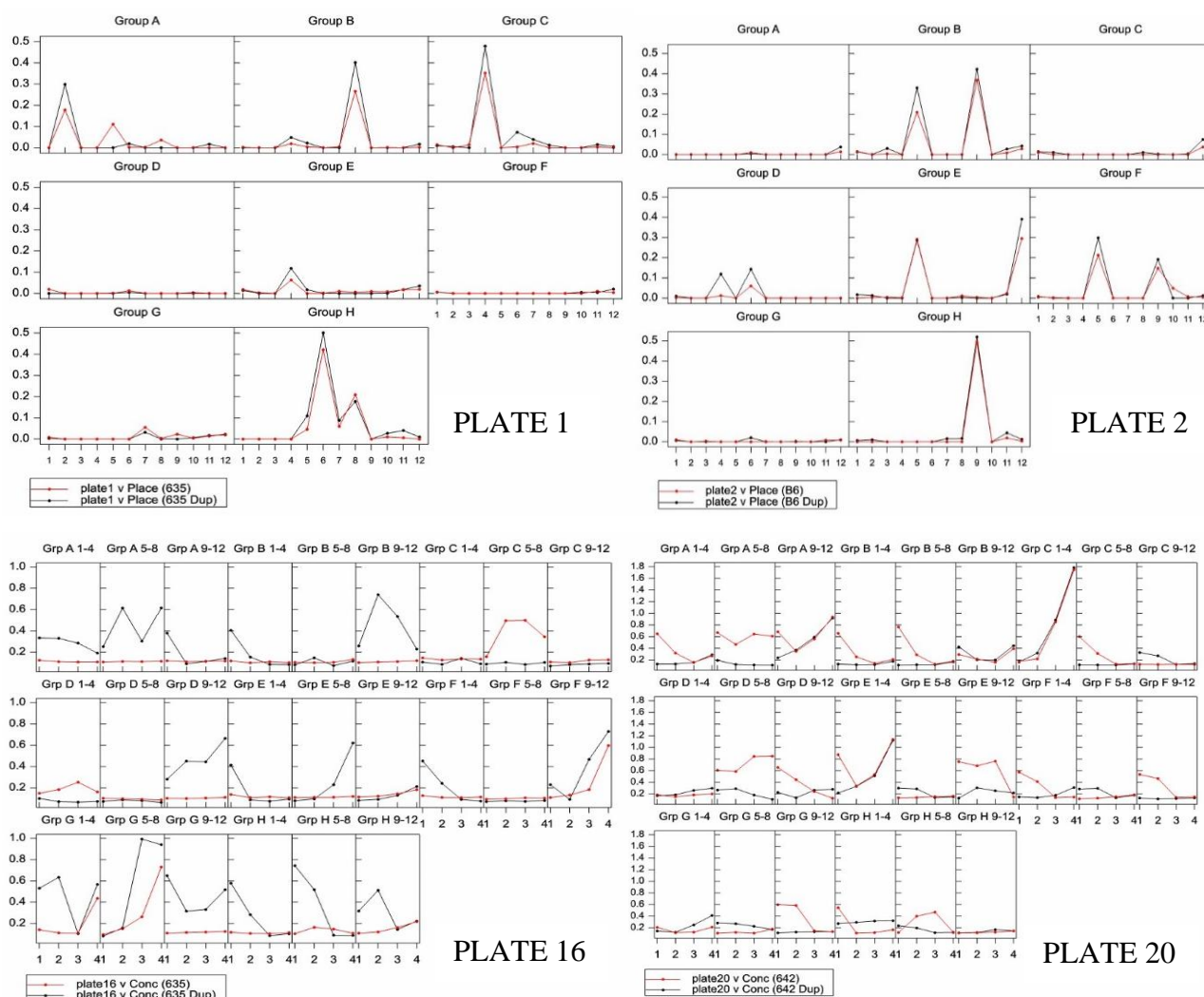


Figure 5. Representative results for *C. fetus* subsp. *venerealis* strains (ATCC19438, 635, B6 and 642-21) tested in duplicate on Biolog PM plates showing higher reproducibility across plates 1 and 2 (carbohydrate sources), with decreasing reproducibility across the remaining plates as seen on plates 16 and 20 (chemical sensitivities). ‘Groups’ are rows A-H across the test plates with numbers 1-12 representing wells across the columns of the plates. Red lines show the initial test result, with the black line showing the results of the repeated testing. The y-axis shows the absorbance reading of the well, while the x-axis is the individual assay on the plate; plates 1 and 2 showing wells 1-12 across the plate rows, and plates 10-20 showing wells 1-12 grouped in 3 lots of 4 wells across each row (e.g. Group G 1-4 = row G well 1 – 4). Substrate identities are shown in Appendix 1.

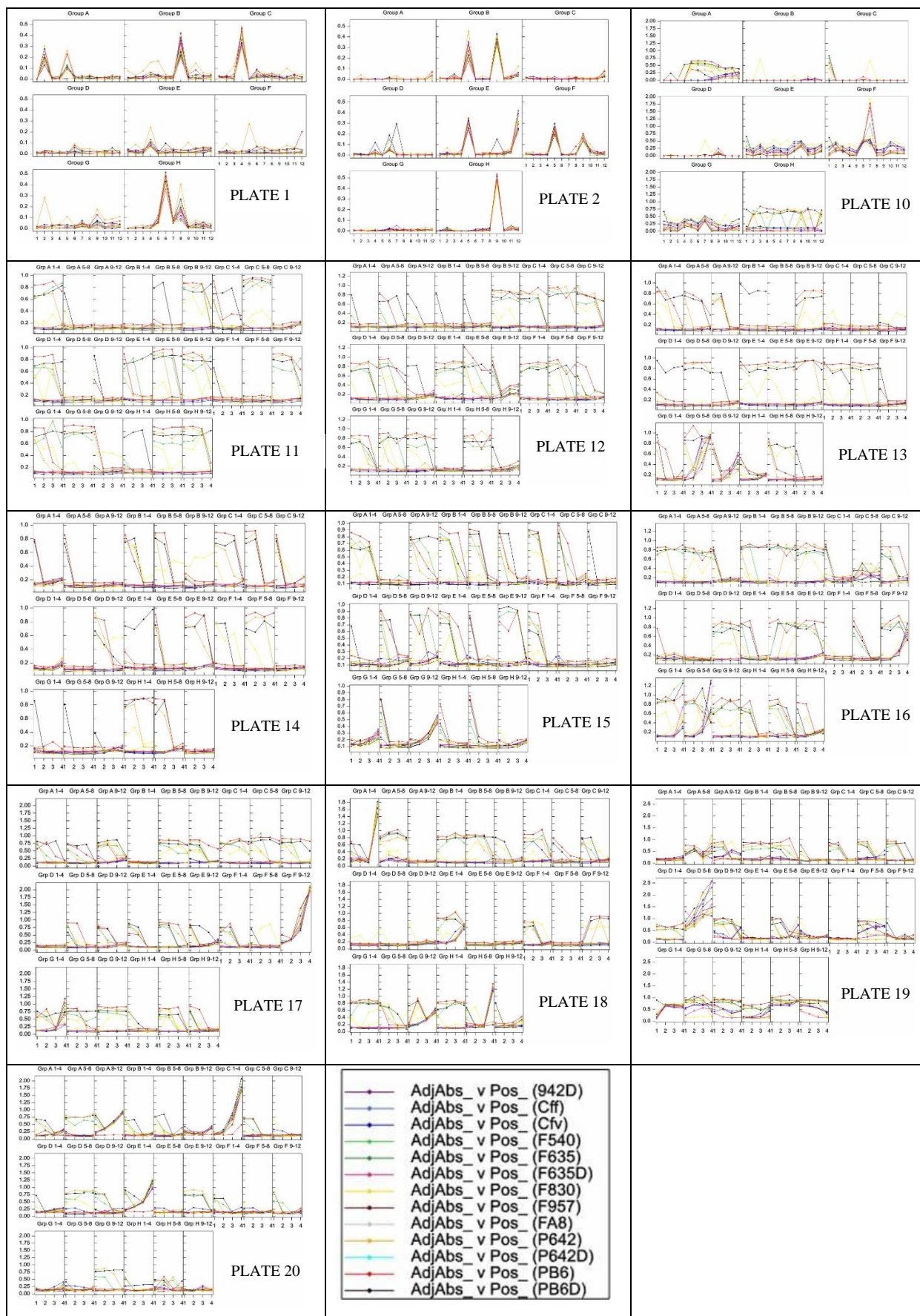


Figure 6. Diagrammatic representation of results for 7 *C. fetus* subsp. *venerealis* strains (A8, B6, 540, 635, 642, 924 and 957) as well as one *C. hyointestinalis* strain (830) and a reference *C. fetus* subsp. *fetus* (ATCC25936) and *C. fetus* subsp. *venerealis* (ATCC19438) strain with duplicates shown with a 'D' after the isolate number, across the 13 plates tested. Each plate shows the adjusted absorbance reading (absorbance minus the negative control well reading) for each well per plate. Wells are represented as 1-12 across rows A-H or in three groups of 1-4 (defining one test category on the plate) across the rows.

There was a high level of variation in substrate respiration between the six singularly tested *Campylobacter* isolates; *C. fetus* subsp. *fetus* ATCC25936, *C. fetus* subsp. *venerealis* A8, 540, 830, 924 and 957. Again, dissimilarity in species test results was more evident across the pH and chemical sensitivity plates (plates 10 – 20) (Figure 2). There did not appear to be any distinguishing results between the tested *C. fetus* subsp. *venerealis* strains and the *C. hyointestinalis* (830) or reference *C. fetus* subsp. *fetus* (ATCC25936) strain.

5.5 Discussion

This chapter addressed the evaluation of the available methods for phenotypic characterisation of isolated *Campylobacter* cultures from bull preputial or heifer vaginal mucus samples. This was done using culture phenotyping as well as API and Biolog as modern phenotyping methods to differentiate species, subspecies and biovars. It was found that culture phenotyping according to OIE recommended guidelines is still the most reliable method for isolate identification. Expanding on antibiotic profiling could provide promising assays for further support in differentiation of species and subspecies. API kit testing had a low identification rate for *C. fetus* subsp. *venerealis* strains, with a large proportion of false negative identifications. Biolog technologies had poor reproducibility and with the lack of an available comparative database for *C. fetus* results, the test is not useful in a diagnostic capacity.

Culture phenotyping of isolated *Campylobacter* organisms using OIE recommended biochemical tests identified a high proportion of non-*C. fetus* subsp. *venerealis* isolates in the collection. This is surprising considering the specific enrichment and isolation methodologies used to favour its culture. A high number of isolates (n=65) were unable to be characterised, due predominantly to varying oxidase results. In order to maintain laboratory characterisation protocols, single isolated colonies were used to perform oxidase testing where possible, and to maintain a consistent inoculum size across all tested isolates. However, it is possible that a higher inoculum is required to perform the oxidase test, which would require investigation into the possibility of false positives before implementation. Previous research has shown that higher concentration inocula have produced false positive results for biochemical testing of *Campylobacter* species, with 10^6 CFU/ml suggested as the ideal, reproducible concentration for tests including sodium chloride or glycine tolerance (On and Holmes 1991). While the presence of variable test results in the biochemical differentiation table allows for identification of strains with deviations in specific test results, its limitation is the possibility of identifying a single strain as multiple species, as is seen with several *C. jejuni* strains. However, removing the variable test results may produce false negatives by not allowing for the identification of anomalous strains. Therefore, while biochemical characterisation is important, it may be prudent for caution to be applied when interpreting results. Clinical history should be considered in diagnosis and if possible suspect isolates should be submitted to molecular confirmation (PCR, 16S rRNA, MLST).

All isolates shared from the bull prepuce survey (n=54) were isolated and identified as *C. fetus* subsp. *venerealis* using molecular techniques including Hum and McMillen PCR profiling (Indjein

2013). However, biochemical characterisation of those strains identified only 35.2% (n=19) as *C. fetus* subsp. *venerealis*. Again, issues with certain biochemical testing methodologies may account for possible false negative results. These include laboratory procedural disparities that need to be noted in protocols where the assumption that laboratories perform basic biochemical tests to the same method may not always be true. Also, awareness of the potential variation of *Campylobacter* species isolated from bull penile sheaths needs to be further studied to ensure that all species are considered during the isolation and differentiation process. This may also help in understanding the increased presence of non-*C. fetus* subsp. *venerealis* isolates (usually thermophilic) recovered from diagnostic samples in Queensland, which has not been previously reported.

Isolates of *C. fetus* showed variable resistance to antibiotics included in media used for transportation and isolation (trimethoprim and polymyxin B). Due to a lack of recommended guidelines for antibiotic interpretations for *C. fetus*, zones of inhibition have been noted, instead of interpretation of sensitivity. All strains were resistant to trimethoprim, however, all strains produced zones of inhibition with polymyxin B. This susceptibility to polymyxin B has been previously mentioned in the literature (Jones, Davis et al. 1985), and this finding led to the subsequent decrease in the concentration of polymyxin B in Lander's Transport and Enrichment Medium (Hum, Hornitzky et al. 2009). Streptomycin showed large zones of inhibition with all tested *C. fetus* strains. It was commonly used a treatment for BGC in cattle, though is no longer a treatment option due to reduced usages in food production animals (NRA 1999). While only a small subset of strains from three species (*C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis* and *C. hyointestinalis*) (n=6) were tested, a number of antibiotics showed zone variations of interest that could be useful for the differentiation of *C. fetus* subspecies if results are able to be extrapolated to a larger number of strains from each species. Antibiotic differentiation between *C. fetus* and *C. hyointestinalis* could be further investigated as currently protocols, such as OIE or ANZSDP (Australian and New Zealand Standard Diagnostic Procedure), for differentiation of *Campylobacter* species use nalidixic acid only (Hum, Hornitzky et al. 2009, OIE 2012). This reliance on a single antibiotic which is variable for *C. fetus* subsp. *venerealis* and resistant for both *C. fetus* subsp. *fetus* and *C. hyointestinalis* does not offer a reliable level of differentiation between species. These antibiotic trends could be further used to optimize media for the enrichment and isolation of *C. fetus* subsp. *venerealis*. Antibiotic substrate respiration results on the GEN III microplate showed that all *C. fetus* subsp. *venerealis* strains (n=16) were able to catabolise vancomycin which is also included in transport and isolation media, while other *Campylobacter* species were not consistent over the strains tested.

While our study was limited in the number of *C. fetus* subsp. *venerealis* strains tested (n=44), resistance was found to one strain against tetracycline and two strains against streptomycin. These results may be indicative of geographical differences in antimicrobial susceptibilities that should be further investigated. There is minimal literature regarding antimicrobial susceptibility in *C. fetus* subsp. *venerealis* strains, with only one previous study describing the susceptibility of 50 German *C. fetus* subsp. *venerealis* strains to eight antibiotics commonly used in extenders in preparation of semen for artificial insemination (Hanel, Hotzel et al. 2011). The study found that there was a low level of antimicrobial resistance, with only seven strains displaying resistance to a combination of the tested antibiotics; spectinomycin (n=4), lincomycin (n=4), streptomycin (n=2), ciprofloxacin (n=1), erythromycin (n=1) and tetracycline (n=1). The antimicrobial contents of transport and enrichment media may need to be altered to reflect not only other contaminating bacteria, but *Campylobacter* species which may be contending for isolation as well as variations in antibiotic susceptibilities.

API Campy kits were used to identify 42 strains of four different species; *C. fetus* subsp. *venerealis*, *C. hyointestinalis*, *C. sputorum* and *Arcobacter cryaerophilus* compared to culture phenotyping. The kits did not produce a high agreement between culture phenotyping and its automated result of *C. fetus* subsp. *venerealis* strains (50.0%). This is not a sufficient characterisation rate for diagnostic identification and should not be relied upon. The assay did however correctly identify the three *Arcobacter cryaerophilus* strains included, however, also produced false *Arcobacter* positive results for other *Campylobacter* strains. While this is not a large sample size, further investigations into *Arcobacter* identification and differentiation could ascertain whether it may be a useful tool for diagnostic laboratories. It is evident in the comparison of the API Campy kit and culture phenotyping that the latter performs better by correctly identifying a larger variety of species, even in the presence of methodological deviations.

Biolog GEN III was used to characterise 27 strains of five different species; *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus*, *C. jejuni*, *C. hyointestinalis* and *C. sputorum*. Biolog GEN III could be compared to API kits or culture phenotyping as it is marketed for the identification of bacterial strains. Biolog PM was used for the characterisation of 10 strains; 8 *C. fetus* subsp. *venerealis*, one *C. fetus* subsp. *fetus* and one *C. hyointestinalis* with the aim of identifying defining characteristics over a range of substrates. Biolog PM is more useful for the characterisation of phenotypic characteristics and the relation to genotyping with catabolism of strains being assessed. Due to this being a preliminary study of the system and budget constraints, reading of Biolog PM

plates was done using a spectrophotometer with raw data analysed using statistical software programs which was a lot more time consuming and labour intensive. Either test is likely to be more user friendly with the correct hardware and software for reading and analysing the results. Biolog GEN III plates were analysed using the Omnilog data program, however, as no *Campylobacter* species are included in the database, the end result was not of primary importance compared to the individual test results. The identification of methyl pyruvate which appeared to correlate to *C. fetus* subsp. *venerealis* bv. Intermedius strains requires further investigation with more strains. The involvement of methyl pyruvate in the cysteine cycle of *Campylobacter* species has not been previously recognised, and may provide a different means of testing the same biochemical characteristic. Cysteine is able to be metabolised to pyruvate or hydrogen sulphide by different pathways (Figure 7) which may make them interchangeable assays for detection of *C. fetus* subsp. *venerealis* bv. Intermedius strains.

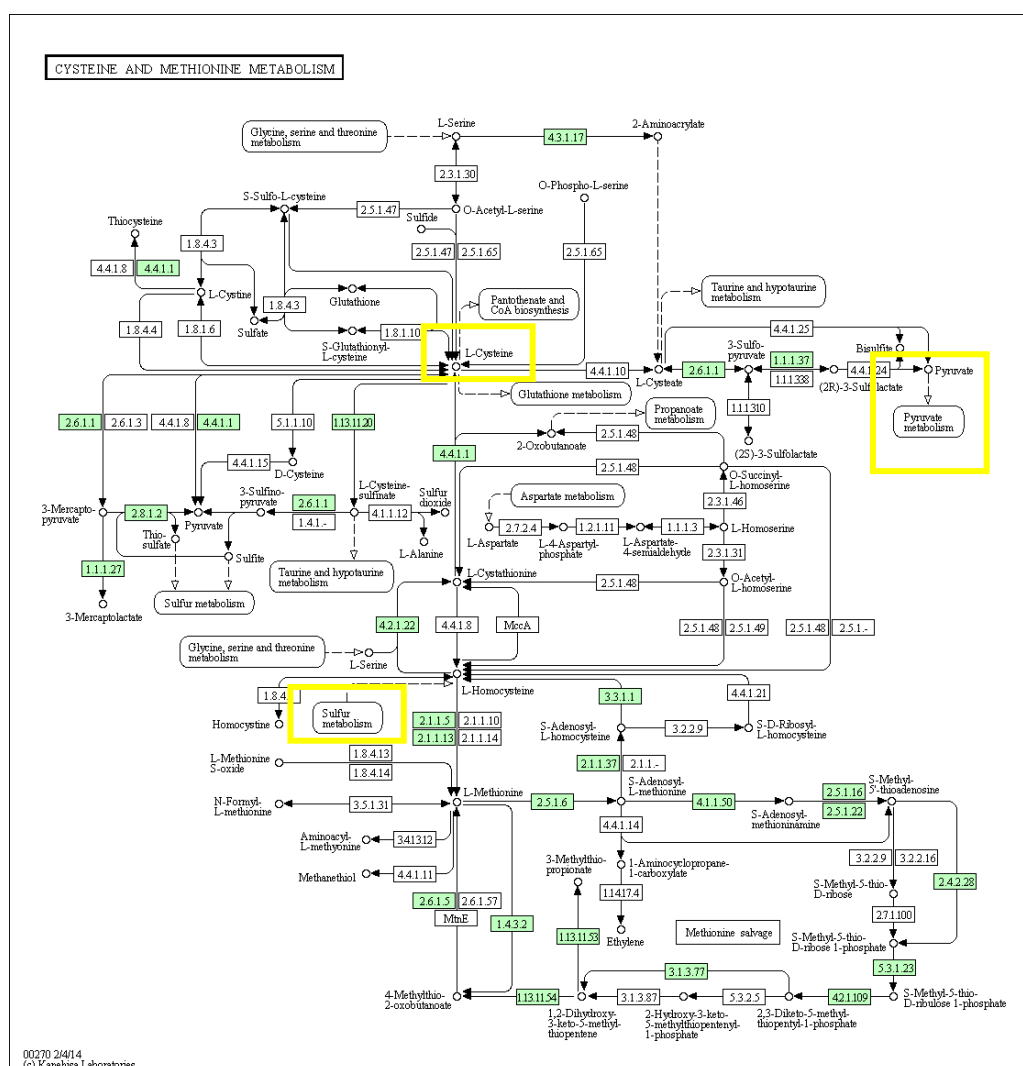


Figure 7. Cysteine and methionine metabolism pathway for *C. fetus* subsp. *venerealis* biovar Intermedius strain 03/293 as shown on (http://www.genome.jp/kegg-bin/show_pathway?cfv00270)

Biolog PMs measures respiration rates of bacterial species, as opposed to growth, to allow a more sensitive detection and analysis of phenotypes (Bochner 2009). Previous problems noted with identification of *C. fetus* have been due to the asaccharolytic nature of the bacteria with inability to oxidise or ferment a variety of carbon substrates (On 1996). Although it has been shown that there was respiration across most of the carbon sources included in the Gen III microplates, whether any of these could be used for media improvements would depend on further studies investigating the reproducibility of these results, as well as the consistency of substrate utilisation across species, subspecies and biovars.

Biolog PMs showed variable reproducibility across the three duplicated *C. fetus* subsp. *venerealis* strains, with a high level of correlation with respect to carbohydrate sources compared to other substrates. Duplications were carried out at two different time points, with strain inocula made to the same specifications though it is speculated that genomic changes in the bacteria may have caused them to vary across pH and chemical substrate tests. It is possible that the carbon utilization genes are more stable and therefore even after resuscitation and retesting, the strains still produced a similar result with these substrates. While all precautions were taken to ensure duplicated inocula were produced in exactly the same manner, slight variations in growth phase, turbidimeter readings or incubation conditions may have influenced the results. The inability to identify individual assays that may differentiate between *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* or *C. hyointestinalis* is partly due to the low number of strains tested for comparing species. A larger number of strains would need to be assessed to determine the specific profiles for each species, before comparisons could be made.

Culture phenotyping according to OIE methodology still remains the most reliable method for characterisation of strains compared to API Campy kit or Biolog GEN III testing. API and GEN III kits showed that they were unable to distinguish between species and subspecies based on the assays included or the identification databases available. There were a high number of false negative results for *C. fetus* subsp. *venerealis* and false positives for *Arcobacter* identification with API kits. GEN III microplates did not show any differentiating results between species and without the availability of a *Campylobacter* database its use for identification purposes are limited. If these two technologies could include more *C. fetus* profiles based on a wide range of strains, future studies may find these more useful with potential for inclusion in diagnostic testing. The identification and differentiation of strains needs to be more specifically outlined in protocols with

positive and negative controls defined as necessary to reduce as much discrepancy as possible with strain testing across different laboratories. A number of differentiating characteristics needs to be identified so that more robust, repeatable tests can be implemented. Antibiotic profiling as a means of differentiation could offer a complementary set of tests if further research found consistent variations in sensitivities of different species and subspecies. It could also assist in improving transport and isolation media to help reduce contamination from non-pathogenic *Campylobacter* species as well as identify geographical variations that may account for differences in species isolations (thermophilic particularly) in tropical countries such as Australia.

Table 17. Supplementary results for all 152 *Campylobacter* strains biochemically profiled in this chapter against OIE recommended tests as well as aerobic growth and susceptibility to tetracycline (30 µg) and streptomycin (10 µg). Isolate numbers 1 – 54 were isolated from a bull prepuce survey (Indjein 2013); number 55 and 56 are DAF ATCC strains; number 57 – 70 are DAF diagnostic isolations; number 71 – 144 were isolated during this PhD from Queensland herd surveys; number 145 and 146 are biovar reference strains; number 147 – 150 are strains shared from the UK Weybridge diagnostic lab and number 151 – 152 are ATCC strains. MLST identification was conducted as part of Ms. Y. Nordin's honours thesis (Nordin 2013). Any mismatches between biochemical and MLST identifications are noted with and * next to the isolate number.

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
1	10	1	1	1	0	1	0	1	1	0	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
2	130	1	0	1	0	1	1	1	0	0	0	0	0	no ID	<i>Bacteroides ureolyticus</i>
3	154b	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
4	158	1	0	1	0	0	1	1	1	1	0	0	0	no ID	<i>Arcobacter</i> sp.
5	166	1	0	1	0	0	1	1	0	1	0	0	0	no ID	<i>Arcobacter</i> sp.
6	214	1	1	1	0	1	0	1	1	0	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
7	217	1	1	1	0	1	0	1	1	0	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
8	218	1	0	1	0	0	0	1	0	0	0	1	0	no ID	<i>Arcobacter</i> sp.
9	227	1	0	1	0	0	1	1	1	1	0	0	0	no ID	<i>C. fetus</i> subsp. <i>venerealis</i>
10	229	1	1	1	0	1	0	1	1	0	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
11	258	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
12	273	1	1	1	0	0	1	1	1	0	0	0	0	no ID	<i>Arcobacter</i> sp.
13	289	1	1	1	0	1	0	1	1	1	0	0	0	no ID	<i>Arcobacter</i> sp.
14	305	1	1	1	0	0	1	1	1	1	0	0	0	<i>Arcobacter</i> sp.	<i>Arcobacter</i> sp.
15	306	1	1	1	0	0	1	1	1	1	0	0	0	<i>Arcobacter</i> sp.	<i>Arcobacter</i> sp.
16	312	1	0	1	0	0	1	1	1	1	0	0	0	no ID	<i>Arcobacter</i> sp.
17	317	1	0	1	0	1	0	1	1	0	0	0	0	no ID	<i>Bacteroides ureolyticus</i>
18	337	1	1	1	1	1	0	1	0	1	0	1	0	<i>C. hyointestinalis</i>	<i>C. hyointestinalis</i>
19	344	1	1	1	0	0	1	1	1	0	0	0	0	no ID	<i>Arcobacter</i> sp.
20	347	1	1	1	0	1	0	1	1	1	0	0	0	no ID	<i>Arcobacter</i> sp.
21	385b	1	1	1	1	1	0	1	1	1	0	0	1	<i>C. hyointestinalis</i>	ND
22	387	1	0	1	0	1	0	1	1	0	0	1	1	no ID	ND
23	400b	1	1	1	0	1	0	1	1	0	0	0	0	<i>Campylobacter fetus</i> subsp. <i>venerealis</i> bv. <i>Venerealis</i>	ND
24	412b	1	1	1	0	1	0	1	1	0	0	0	0	<i>Campylobacter fetus</i> subsp. <i>venerealis</i> bv. <i>Venerealis</i>	ND
25	430	1	1	1	0	0	0	1	1	0	0	0	0	no ID	uncultured genome
26	475	1	0	1	0	1	0	1	1	0	0	0	0	no ID	<i>C. fetus</i> subsp. <i>venerealis</i>
27*	520	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	did not amplify
28	521	0	0	0	0	1	0	1	1	1	0	0	0	no ID	did not amplify
29	530b	1	1	0	1	1	1	1	1	0	1	1	1	<i>C. sputorum</i>	ND
30	531 ^b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
31	534 ^{b,c}	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
32	537b	1	1	0	0	1	1	1	1	0	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. <i>Intermedius</i> [^]	<i>C. fetus</i> subsp. <i>venerealis</i>
33	540 ^{a,b,c,d}	1	1	1	0	1	0	1	1	0	0	0	V	<i>C. fetus</i> subsp. <i>venerealis</i> bv.	<i>C. fetus</i> subsp. <i>venerealis</i>

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
														Intermedius	
34*	628	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. hyointestinalis</i>
35	635 ^{a,b,c,d}	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>
36	672 ^b	1	1	1	0	0	1	1	0	1	0	0	0	<i>Arcobacter</i> sp.	<i>Arcobacter</i> sp.
37	673 ^b	1	1	1	0	1	1	1	1	1	0	0	0	<i>Arcobacter</i> sp.	<i>Arcobacter</i> sp.
38	676 ^{b,c}	1	1	1	1	1	0	1	1	0	0	0	1	<i>C. hyointestinalis</i>	<i>C. hyointestinalis</i>
39	729 ^b	1	1	1	0	1	0	1	1	1	0	0	0	no ID	<i>Arcobacter</i> sp.
40	742 ^{b,c}	1	1	1	0	1	0	1	1	0	0	0	V	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	ND
41	744 ^{b,c}	1	1	1	0	0	0	1	1	0	0	0	1	no ID	<i>C. hyointestinalis</i>
42	830 ^{a,b,c,d}	1	1	1	V	1	0	1	1	0	0	0	V	<i>C. hyointestinalis</i>	<i>C. hyointestinalis</i>
43	849 ^{b,c}	1	0	0	1	1	0	1	1	0	0	1	1	no ID	<i>C. sputorum</i>
44	863 ^{b,c}	1	0	0	1	1	0	1	1	0	0	1	1	no ID	<i>C. sputorum</i>
45	882 ^b	1	1	1	1	1	0	1	1	0	0	1	0	<i>C. hyointestinalis</i>	<i>C. hyointestinalis</i>
46	890	1	1	1	0	0	0	1	1	0	0	0	1	no ID	<i>C. hyointestinalis</i>
47	923 ^b	1	1	1	0	1	1	1	1	0	0	0	w	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
48	924 ^{b,c,d}	1	1	1	0	1	0	0	0	0	0	0	V	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
49	926 ^{b,c}	0	1	1	0	1	1	1	1	0	0	0	w	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
50	951 ^b	1	1	1	0	1	0	1	1	0	0	0	V	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
51	957 ^{b,c,d}	1	1	0	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis ^	<i>C. fetus</i> subsp. <i>venerealis</i>
52	979 ^{b,c}	1	1	1	1	1	0	1	1	0	0	1	1	<i>C. fetus</i> subsp. <i>fetus</i> / <i>C. hyointestinalis</i>	<i>C. hyointestinalis</i>
53*	1036	1	1	0	0	0	0	0	0	0	0	0	0	<i>C. jejuni</i>	<i>C. hyointestinalis</i>
54	BECKS	1	1	1	0	1	0	1	0	1	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	ND
55	DAF - Q6 ^c	1	1	0	1	ND	ND	ND	ND	0	0	0	0	<i>C. jejuni</i> / <i>C. hyointestinalis</i>	ND
56	DAF - Q41 ^c	1	1	1	0	1	0	1	1	0	0	0	0	<i>Campylobacter fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>
57	DAF - 3329	1	1	1	0	1	0	1	1	0	0	0	0	<i>Campylobacter fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
58	DAF – 75838 ^c	1	1	1	0	1	1	ND	ND	ND	0	0	0	<i>Campylobacter fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
59	DAF – 76223 ^c	1	1	1	0	1	1	1	1	0	0	0	V	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
60	DAF – 101302 ^b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. spitorum</i>	ND
61	DAF - 12581/3	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
62	DAF - 5-46423	1	0	1	1	0	1	1	1	0	1	0	1	no ID	ND
63	DAF - 76107	1	1	0	1	0	0	1	0	0	ND	1	1	<i>C. hyointestinalis</i>	ND
64	DAF - 85-826	1	0	1	1	1	0	0	1	0	0	1	1	no ID	ND
65*	DAF - 87-29394	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>Arcobacter</i> sp.
66*	DAF - F591/1	1	1	1	1	1	0	1	1	1	0	0	0	<i>C. hyointestinalis</i>	<i>C. fetus</i> subsp. <i>fetus</i>
67	DAF - M08/16749 ^c	1	1	0	0	1	1	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv.	ND

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
														Venerealis ^	
68	DAF - M09/10806 ^c	1	1	0	0	1	1	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis ^	ND
69	DAF - W1111	1	1	1	1	1	0	1	1	0	0	0	0	<i>C. hyointestinalis</i>	ND
70	DAF - W1179	1	1	0	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis ^	ND
71	Phd 2 -1	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
72	Phd 2 -2	1	1	1	1	1	0	1	1	0	1	1	1	<i>C. hyointestinalis</i>	ND
73	Phd 2 -3	0	1	1	1	1	0	1	1	0	1	1	1	no ID	ND
74	Phd 2 -4	0	1	1	1	1	0	1	1	0	1	1	0	no ID	ND
75	Phd 2 -5	0	1	1	1	1	0	1	1	1	0	1	0	no ID	ND
76	Phd 2 -6	1	0	1	0	0	1	1	1	0	0	0	0	no ID	ND
77	Phd 2 -7	0	1	1	0	1	0	1	1	0	0	1	0	no ID	ND
78	Phd 2 -8	1	1	1	0	0	0	1	0	0	0	1	0	no ID	ND
79	Phd 2 -9	1	0	1	0	0	1	1	1	0	0	0	0	no ID	ND
80	Phd 2 -10	1	0	1	0	0	1	0	0	0	0	1	1	no ID	ND
81	Phd 2 -11	ND	ND	1	0	0	1	1	1	ND	0	1	0	no ID	ND
82	Phd 2 -12	0	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
83	Phd 2 -13	1	0	1	0	0	0	0	0	1	0	0	0	no ID	ND
84	Phd 2 -14	1	0	1	0	1	1	1	1	1	0	0	0	no ID	ND
85	Phd 2 -15	1	0	1	0	0	1	0	0	1	0	1	1	no ID	ND
86	Phd 2 -16	1	0	1	0	0	1	1	0	1	0	0	0	no ID	ND
87	Phd 2 -17	1	1	1	0	0	0	1	1	0	0	0	0	no ID	ND
88	Phd 2 -18	ND	ND	1	0	0	1	1	1	ND	0	0	0	no ID	ND

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
89	Phd 2 -19	1	0	1	0	0	0	0	1	1	0	1	0	no ID	ND
90	Phd 2 -20	1	0	1	0	0	1	0	1	1	0	1	0	no ID	ND
91	Phd 2 -21	1	0	1	0	1	1	1	1	1	0	0	0	no ID	ND
92	Phd 2 -22	1	1	0	0	1	0	1	1	ND	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis ^	ND
93	Phd 2 -23	1	0	1	0	0	0	0	0	1	0	1	1	no ID	ND
94	Phd 2 -24	0	1	0	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis ^	ND
95	Phd 2 -25	1	1	1	0	1	0	1	1	ND	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
96	Phd 2 -26	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
97	Phd 2 -27	0	1	1	1	1	0	1	1	0	0	1	0	no ID	ND
98	Phd 2 -28	0	1	1	1	1	0	0	1	0	0	1	0	no ID	ND
99	Phd 2 -29	1	0	1	0	0	1	1	1	1	0	1	0	no ID	ND
100	Phd 2 -30	0	1	0	0	1	0	0	0	0	1	0	0	no ID	ND
101	Phd 2 -31	0	1	1	0	1	0	1	1	0	0	1	0	no ID	ND
102	Phd 2 -32/ A8 ^{c,d}	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>
103	Phd 2 -33	1	0	1	0	ND	0	1	1	0	0	1	0	no ID	ND
104	Phd 2 -34	0	1	1	0	1	1	1	1	0	1	1	1	no ID	ND
105	Phd 2 -35	1	0	1	0	0	1	1	0	1	0	0	0	no ID	ND
106	Phd 2 -36	0	1	0	1	1	0	0	0	0	1	1	1	<i>C. sputorum</i>	ND
107	Phd 2 -37	0	1	0	1	1	0	0	ND	0	1	1	1	<i>C. sputorum</i>	ND
108	Phd 2 -38	0	0	1	0	1	0	1	1	0	0	0	1	no ID	ND
109	Phd 2 -39	1	1	1	0	1	0	1	1	0	0	1	1	<i>C. fetus</i> subsp. <i>fetus</i>	ND

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
110	Phd 2 -40	0	1	0	0	0	0	0	0	0	1	0	0	no ID	ND
111	Phd 2 -41	0	1	0	1	1	0	0	0	0	1	1	0	<i>C. sputorum</i>	ND
112	Phd 2 -42	1	1	1	0	0	0	1	0	0	0	1	0	no ID	ND
113	Phd 2 -43/ K8	1	1	1	0	1	0	1	1	0	0	0	1	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	ND
114	Phd 2 -44	0	1	1	1	0	0	1	0	1	1	1	0	no ID	ND
115	Phd 2 -45	0	1	1	0	0	0	1	1	1	0	1	0	no ID	ND
116	Phd 2 -46	0	0	0	1	0	0	0	0	1	0	1	0	no ID	ND
117	Phd 2 -47	0	1	1	1	ND	ND	ND	ND	1	1	1	1	no ID	ND
118	Phd 2 -48	1	0	1	0	0	1	0	1	1	0	1	0	no ID	ND
119	Phd 2 -49	1	0	1	0	0	1	1	1	0	0	1	0	no ID	ND
120	Phd 2 -50	1	0	1	0	0	0	0	1	1	0	1	0	no ID	ND
121	Phd 2 -51	1	0	1	0	0	1	1	1	0	0	1	0	no ID	ND
122	Phd 2 -52	0	0	1	0	1	0	1	1	0	0	0	0	no ID	ND
123	Phd 2 -53	1	0	1	0	0	1	1	1	1	0	1	0	no ID	ND
124	Phd 2 -54	1	0	1	0	1	1	1	1	0	0	1	0	no ID	ND
125	Phd 2 -55	0	1	0	1	1	0	1	1	0	0	1	0	<i>C. jejuni</i>	ND
126	Phd 2 -56	0	1	0	1	1	0	1	1	0	0	1	0	<i>C. jejuni</i>	ND
127	Phd 2 -57	0	1	0	1	1	0	1	1	0	0	0	0	<i>C. jejuni</i>	ND
128	Phd 2 -58	0	1	0	0	1	0	1	1	0	0	1	0	<i>C. jejuni</i>	ND
129	Phd 2 -59	0	1	0	1	1	0	1	1	0	0	1	0	<i>C. jejuni</i>	ND
130	Phd 2 -60	0	1	0	1	1	0	1	1	0	0	1	0	<i>C. jejuni</i>	ND
131	Phd 2 -61	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
132	Phd 2 -62	1	1	1	1	1	0	1	1	0	0	0	0	<i>C. hyointestinalis</i>	ND

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
133	Phd 2 -63/ 39-8 ^c	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	ND
134	Phd 2 -64b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
135	Phd 2 -65	1	1	1	1	1	0	1	1	0	1	1	1	<i>C. hyointestinalis</i>	ND
136	Phd 2 -66b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
137	Phd 2 -67b	0	1	0	1	1	0	1	1	0	1	1	0	<i>C. sputorum</i>	ND
138	Phd 2 -68b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
139	Phd 2 -69	0	1	ND	ND	1	0	1	1	ND	ND	ND	ND	no ID	ND
140	Phd 2 -70b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
141	Phd 2 -71	0	1	ND	ND	1	0	1	1	0	ND	ND	ND	no ID	ND
142	Phd 2 -72b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
143	Phd 2 -73b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
144	Phd 2 -74b	0	1	0	1	1	0	1	1	0	1	1	1	<i>C. sputorum</i>	ND
145	B6 ^{a,b,c,d}	1	1	0	0	1	1	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis *	<i>C. fetus</i> subsp. <i>venerealis</i>
146	642-21 ^{a,b,c,d}	1	1	1	0	1	0	1	1	0	0	0	V	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Intermedius	<i>C. fetus</i> subsp. <i>venerealis</i>
147	BT08/04 ^b	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>
148	BT102/00 ^b	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>
149*	BT154/00 ^b	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>fetus</i>
150*	BT277/06 ^b	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>fetus</i>

No.	ISOLATE	CAT	OXD	25°C	42°C	K	NA	T	S	O ₂	TSI	GLY	H ₂ S	Biochemical Identification	MLST Identification
151	ATCC19438 ^{b,c,d}	1	1	1	0	1	0	1	1	0	0	0	0	<i>C. fetus</i> subsp. <i>venerealis</i> bv. Venerealis	<i>C. fetus</i> subsp. <i>venerealis</i>
152	ATCC25936 ^{a,c,d}	1	1	1	0	1	0	1	1	0	0	1	1	<i>C. fetus</i> subsp. <i>fetus</i>	<i>C. fetus</i> subsp. <i>fetus</i>

CAT – catalase; OX – oxidase; K – cephalothin; NA – nalidixic acid, T – tetracycline; S – streptomycin; O₂ – aerobic growth; TSI – triple sugar iron slope; GLY – growth in 1% glycine medium; H₂S – hydrogen sulphide production in a 0.02% cysteine medium; 0 – no growth/ resistant; 1 – growth/ sensitive; ND – not done; no ID – no identification possible using the OIE standard tests; ^also identified as *C. jejuni*; ^a – antibiotic profiled; ^b – API tested; ^c – Biolog gen III

**Chapter 6. Evaluation and histological
examination of a *Campylobacter fetus*
subsp. *venerealis* small animal model**

6.1 Manuscript Information

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The candidate, Ameera Koya, was primarily responsible for the study design and implementation as well as writing the manuscript. The co-authors: Sharon de Wet, Shirley Turner, Judy Cawdell-Smith, Bronwyn Venus, Ristan Greer, Ala Lew-Tabor and Gry Boe-Hansen contributed to the manuscript on various aspects. The contributions of each co-author is listed on page vii of this thesis.

Evaluation and histological examination of a *Campylobacter fetus* subsp. *venerealis* small animal infection model

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C. fetus subspecies *venerealis*, Campylobacteriosis, Placentitis, Bovine, Guinea pig

Abstract

Bovine genital campylobacteriosis (BGC), caused by *Campylobacter fetus* subsp. *venerealis*, is associated with production losses in cattle worldwide. This study aimed to develop a reliable BGC guinea pig model to facilitate future studies of pathogenicity, abortion mechanisms and vaccine efficacy. Seven groups of five pregnant guinea pigs (1 control per group) were inoculated with one of three strains via intra- peritoneal (IP) or intra-vaginal routes. Samples were examined using culture, PCR and histology. Abortions ranged from 0% to 100% and re-isolation of causative bacteria from sampled sites varied with strain, dose of bacteria and time to abortion. Histology indicated metritis and placentitis, suggesting that the bacteria induce inflammation, placental detachment and subsequent abortion. Variation of virulence between strains was observed and determined by culture and abortion rates. IP administration of *C. fetus* subsp. *venerealis* to pregnant guinea pigs is a promising small animal model for the investigation of BGC abortion.

1. Introduction

Bovine genital campylobacteriosis (BGC) caused by *C. fetus* subsp. *venerealis* is a reproductive disease affecting many cattle herds (World Organisation for Animal Health, 2012). It has been associated with production losses in the Australian meat and dairy industry of up to 60% due to abortion and infertility in infected heifers (Clark et al., 1975). The bacterium is often asymptotically carried by bulls, therefore diagnosis, treatment and control measures may be limited. It is venereally transmitted to heifers during mating. Heifers may either remain asymptomatic or experience late term abortions (Hum et al., 2009). This can lead to a continuous cycle of infection and infertility which may affect herd pregnancy rates (Clark, 1971).

Preventative and control measures for BGC exist in the form of controlled mating, antibiotic administration and vaccines. South American studies have found that vaccines are not always efficacious, with variation in protection offered by different vaccines against different strains (Cobo et al., 2003). This may be due to variation in strain virulence which is currently largely unknown. To define the level of pathogenicity and virulence variation present in *C. fetus* subsp. *venerealis* strains and to determine strain pathogenicity, a model for analysing and defining infection parameters is necessary. While in vitro models are much more ethical and financially viable, a greater understanding of the bacterial genes associated with virulence is needed and only single gene mutants can be examined in a single experiment (Kienesberger et al., 2007). In addition, in vitro models can only address one aspect of virulence i.e. epithelial cell invasion.

Guinea pig models have previously been used to assess variation in pathogenicity of *Campylobacter* species and strains including those of veterinary significance (Burrough, 2011; Burrough et al., 2009; Coid et al., 1987; SultanDosa et al., 1983). However, a model for determining *C. fetus* subsp. *venerealis* virulence has not been evaluated since its initial development 60 years ago (Ristic and Morse, 1953), while recent scientific advances (such as PCR) could improve model assessment. The model has not been used for the study of Australian strains, which may have different pathogenic characteristics to those in North America where the original study was undertaken (Ristic and Morse, 1953). An Australian based polymerase chain reaction assay could not differentiate

UK *C. fetus* subspecies correctly hypothesizing that a unique clone was present in the UK (Hum et al., 1997; Willoughby et al., 2005). This may be due to different bacterial environments as well as antibiotic treatments which can impact bacterial genetic variation. The significance of a guinea pig infection model was shown in further studies using a variety of human *Campylobacter* species (but not *C. fetus*) with culture for diagnosis (Coid et al., 1987; Taylor and Bryner, 1984). Recent studies into *C. jejuni* infection models have included the examination of histological findings to substantiate the bacterial involvement in abortion (Burrough et al., 2009, 2011).

This study aimed to develop an animal model to assess variation in strain pathogenicity and subsequent abortions caused by different *C. fetus* subsp. *venerealis* strains. The model was established with accurate, defined parameters for the identification of dose, route and strains of *C. fetus* subsp. *venerealis* for further studies. The model was evaluated at a range of low, medium and high doses and using one of two different infection routes (intra-peritoneal and intra-vaginal). Subsequently culture, PCR and histology were compared to determine the presence of bacteria and the pathological changes associated with infection.

2. Materials and methods

2.1. Ethical statement

This study was approved by the University of Queensland Animal Ethics Committee (SVS070/10).

2.2. Study design

The study was designed as a controlled experimental trial with 35 female guinea pigs (*Cavia porcellus*) obtained from the University of Queensland's guinea pig breeding facility (Gatton campus) allocated to seven groups of five dams. Guinea pigs were assigned to groups in the order they became pregnant. Guinea pigs were housed in floor pens with wood shavings and had access to vitamin C supplemented water (1 g/l) and Barastoc guinea pig pellets (Ridley AgriProducts, Melbourne, Australia) *ad libitum* as well as fresh vegetables daily.

At the commencement of the study, 20 of the guinea pigs were tested for the presence of *C. fetus* subsp. *venerealis* or *Campylobacter*-like organisms (curved rod organisms growing under microaerobic conditions), by swabbing the vaginal area with a sterile cotton swab and placing it in PBS for transport to the lab where it was streaked out onto a sheep-blood agar (SBA) plate (Oxoid Australia, ThermoFisher Scientific, Waltham, MA, USA) and incubated in a microaerobic workstation (Don Whitely Scientific, Shipley, UK) at 37 °C for 72 h.

Abortion (defined as the expulsion of one or more foetuses or placentas) was the primary outcome. Guinea pigs were randomly housed in groups of 6–8 by blindly allocating dams to each pen with one male for mating purposes. They were then assigned to groups as they became pregnant based on closest gestational ages. Pregnant guinea pigs were inoculated via intra-peritoneal (IP) (groups 1–6) or intra-vaginal (IVA) (group 7) route between 5 and 6 weeks of gestation based on retrospective calculations of oestrus detection data. Each group consisted of five animals with an allocation ratio of 4:1 (test: control) per group (control receiving sterile broth).

2.3. *Pregnancy determination*

During the mating period, the guinea pigs were oestrus-detected by assessing the opening of the vaginal membrane (Stockard and Papanicolaou, 1919) once daily. When oestrus had not been detected for 21 consecutive days, trans-abdominal ultrasound using a MyLab 30 Vet ultrasound scanner with a 5–10 MHz linear probe (Esaote Pie Medical, Genoa, Italy) was performed. The presence of embryonic vesicles with foetuses and heartbeats confirmed pregnancy. The first five animals to fall pregnant were used in the first treatment group, with each subsequent set of five animals forming the following six treatment groups.

2.4. *Inoculum preparation*

Three *C. fetus* subsp. *venerealis* isolates were utilized in this study. Strain Q41 (ATCC19438), a control *C. fetus* subsp. *venerealis* was obtained from New South Wales Department of Primary Industries, and strains 258 and 540 were obtained from a bull

prepuce abattoir survey with no traceability of bull infective status (Indjein, 2013). The three strains used were selected based on three features; (1) the molecular and biochemical profile matching *C. fetus* subsp. *venerealis* outlined by the World Organisation for Animal Health (World Organisation for Animal Health, 2012), (2) the viability of the bacteria 72 h after resuscitation based on bacterial growth and motility as seen microscopically, and (3) the survival of the bacteria when inoculated into 5 ml of guinea pig serum and incubated in a microaerobic workstation for 72 h.

The strains were resuscitated from storage at -80°C by placing 200 μl of one of the stored media, either 85% FBP medium (Gorman and Adley, 2004) (0.025% ferrous sulphate w/v (Univar Australia Pty Ltd, Ingleburn, NSW, Australia), 0.025% sodium metabisulphite w/v (Sigma-Aldrich, St. Louis, MO, USA), 0.025% sodium pyruvate w/v (BDH ProLab, VWR International Pty Ltd, Murarrie, QLD, Australia) or 15% glycerol (AnalaR NORMAPUR, VWR International Pty Ltd), into 10 ml of vegetable peptone based Campylobacter broth consisting of 1% vegetable peptone no 1 w/v (Oxoid Australia, ThermoFisher Scientific), 0.2% sodium succinate w/v (Merck Australia, Merck KGaA, Darmstadt, Germany), 0.5% yeast extract w/v (Oxoid Australia, ThermoFisher Scientific), 0.5% sodium chloride w/v (Merck Australia, Merck KGaA), 0.0001% magnesium sulphate w/v (Univar Australia Pty Ltd), 0.5% calcium chloride w/v (Merck Australia, Merck KGaA), and 0.15% bacteriological agar w/v (Oxoid Australia, ThermoFisher Scientific). The broths were placed into a microaerobic workstation at 37°C and assessed at 72 h for growth. Viable strains were profiled using the Hum conventional PCR (Hum et al., 1997) and characterized biochemically according to OIE classification (World Organisation for Animal Health, 2012) – catalase, oxidase and growth in 1% glycine medium (Becton Dickinson and Company, Sparks Glencoe, MD, USA), growth at 42°C , 25°C and aerobically, susceptibility to 30 μg nalidixic acid and cephalothin (Oxoid Australia, ThermoFisher Scientific), H_2S production in a triple-sugar-iron (TSI) slope (Oxoid Australia, ThermoFisher Scientific) and in a 0.02% cysteine medium (Fluka analytical, Sigma-Aldrich) using lead acetate paper (Fluka analytical, Sigma-Aldrich).

The strains identified biochemically as *C. fetus* subsp. *venerealis*, were inoculated into 10 ml of fresh vegetable peptone based Campylobacter broth (as described above)

and incubated microaerobically at 37 °C for 72 h. At 72 h, a serial dilution of 10^{-1} to 10^{-9} CFU/ml was prepared and plated in duplicate by placing 100 µl of the dilution onto an SBA plate and spreading it evenly across the surface. The plates were then incubated in the microaerobic workstation for 72 h. Subsequently, a comparative cell count using a Helber bacterial counting chamber (Hawksley, Sussex, UK) was carried out by creating a 1/10 dilution of the broth in 10% neutral buffered formalin (NBF) (10% w/v formaldehyde, Merck Australia, Merck KGaA) to fix the bacteria. A volume of 10 µl was placed into the central chamber of the counter, the cover slide added and viewed under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) at 400× magnification and the bacterial concentration calculated (Meynell and Meynell, 1970). Concentrations of the inoculum for groups 1–5 and 7 were based on growth at 72 h while group 6 was diluted with Campylobacter broth to achieve the desired concentration of bacterial colonies. Inoculum were streaked out just prior to inoculation onto two SBA plates, incubated at 37°C either microaerobically or aerobically to ensure sterility of the broth.

3. Experimental procedures

3.1. Animal inoculation

IP inoculation with 1.0 ml of the prepared strain was carried out using a 21G needle inserted slightly to the left of the midline of the abdomen after swabbing with iodine.

IVA inoculation was performed by placing the guinea pig on its back, anaesthetizing with gaseous isoflurane using a Universal Vaporizer anaesthetic machine (Universal Vaporizer Support, Foster City, CA, USA) and maintaining by mask with 1–2% isoflurane (Pharmachem, Kearny, NJ, USA) and 2–6 l oxygen per min. The vagina was gently opened using a sterile swab and a 3.0 mm tom-cat catheter (Henry Schein, New York, NY, USA) inserted into the cranial part of the vagina. The inoculum was then injected into the vagina, and the guinea pig maintained under anaesthesia for a further 5 min.

3.2. Animal monitoring

Following inoculation, the animals were placed into individual pens and were checked twice daily (AM and PM) for any signs of systemic illness, such as increase in temperature or respiration, vaginal opening, bleeding, or abortion. An abdominal ultrasound was conducted every day (AM) to determine number and health of foetuses, including movement and foetal heartbeat.

3.3. Experimental outcomes

The primary outcome was time to abortion or time to euthanasia if no abortion had occurred by the end of the study period. Animals displaying vaginal bleeding without foetal death or expulsion of a foetus were closely monitored but were not considered to be aborting. Upon the occurrence of an abortion event (expulsion of one or more foetuses or placentas), the dam was anaesthetized, sampled and euthanized.

Secondary outcomes were bacterial culture; evidence of the bacterium in tissues detected by PCR and histological evidence of infection in the reproductive tract, including metritis or placentitis.

3.4. Sampling protocol

Immediately upon the detection of an abortion event or at the end of the experiment timeline had no abortions occurred, tissues were sampled for bacteria. Each guinea pig was anaesthetized with gaseous isoflurane and maintained by mask with 1–2% isoflurane and 2–6 l oxygen per minute. The abdominal area was swabbed with iodine and rear foot reflexes and breathing monitored before commencing with the sampling process. A longitudinal cut was made in the abdominal mid-line and the peritoneal cavity was swabbed. The left uterine horn was exteriorized and sampled using a swab through a small incision into the uterus. The uterine horn was then incised and the utero-placental junctions were swabbed. The foetuses in the horn were removed with the placenta and attachment site intact (incising through the uterine wall), and placed into individual petri dishes.

If the foetus had been expelled, the placenta with the attachment site was swabbed. The

sampling protocol was then repeated for the right uterus horn. Due to the time sensitive nature of the procedure, plating could not be carried out at the time of sampling. To ensure the viability of the organism, all swabs were placed in an enrichment medium, Lander's (2.1% Mueller-Hinton broth w/v (Oxoid Australia, ThermoFisher Scientific), 0.5% bacteriological charcoal w/v (Amyl Media, Kings Langley, NSW, Australia), 7% lysed horse blood v/v (Oxoid Australia, ThermoFisher Scientific), 0.4% campylobacter growth supplement v/v (Oxoid Australia, ThermoFisher Scientific), 0.01% cyclohexamide w/v, 0.002% vancomycin w/v, 0.001% trimethoprim w/v and 2500 IU polymyxin B w/v (Sigma Aldrich)) until delivery to the laboratory (~85 km).

Cardiac puncture of the dam was performed and two drops of blood were placed directly into Lander's transport medium for culture, while the remaining volume was placed into 9 ml culture tubes for extraction of serum for PCR. Immediately following cardiac puncture the guinea pig was euthanized with 2 ml of Lethobarb (Virbac, Carros, France). Amniotic fluid (AF) was collected and the foetus was incised to remove the foetal spleen and collect foetal stomach contents (FSC). AF and FSC samples were placed into individual Lander's media. The placentas, foetuses and a 2 cm² piece of uterus tissue were placed into individual containers of 10% NBF for histology and a 0.5 cm² piece of the uterus horn and the spleens from the foetuses and dam were placed in individual microtubes on ice for PCR.

3.5. *Bacterial culture*

Samples collected in Lander's transport medium were incubated overnight at 37 °C before streaking onto SBA plates and assessed at 72 h for growth of *Campylobacter*-like colonies. These colonies were then biochemically tested according to international standards as published in the World Organisation for Animal Health manual (2012) to ensure the identity matched the biochemical profile of the inoculum.

3.6. *PCR analysis*

The PCR previously described by Hum and colleagues (Hum et al., 1997) was carried out on DNA prepared from maternal and foetal spleen and dam sera from group 4 to 7, as well as a piece of the right uterus horn and each placenta from group 5 to 7. Tissue digestion and extraction was carried out using a Qiagen tissue kit (Qiagen, Hilden, Germany). Hum assay PCR primers were synthesized through Sigma (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) and PCR assays were undertaken in 20 µl reaction volumes using PCR reaction buffer with 25 mM Mg²⁺ (10 × Hotmaster™ Taq buffer, 5 Prime, Quantum Scientific, Lane Cove West, NSW, Australia), 1U Hotmaster™ Taq DNA polymerase (5 Prime, Quantum Scientific), 1 µM of each forward and reverse primers, 1 µM dNTPs (Invitrogen) and 1.25 ng of *C. fetus* subsp. *venerealis* genomic DNA. The reactions were amplified in a DNA Engine Thermal Cycler (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) using the following parameters: initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation for 20 s, annealing at 50 °C for 20 s, and extension at 72 °C for 2 min including a final extension of 10 min at the end. Products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA, pH 8) agarose gels containing 1% GelRed™ Nucleic Acid Gel Stain (Biotium, Jomar Diagnostics P/L, Stepney, SA, Australia) using either MassRuler™ Low range DNA ladder (Fermentas, ThermoFisher Scientific) or HyperLadder I (Bioline, Alexandria, NSW, Australia) and were visualized under Trans-UV light using the GelDoc™ XR+ system (Bio-Rad Laboratories Pty Ltd).

3.7. *Histological analysis*

All fixed tissue samples were trimmed through the centre-most point to allow maximum area for visualization. Placentas attached to the uterus were trimmed to allow assessment of the utero-placental junction as well as the centre of the placenta (interlobium, labyrinth and subplacenta). Foetuses were sectioned three times, through the head, thorax and abdomen. While amniotic sac samples were collected when available, due to the low number these data are omitted. The sections were then embedded in paraffin and routinely processed for haematoxylin and eosin (H&E) staining. Histologically, neutrophil aggregation and the presence of suppurative exudate, the first indicators of infection, were used to define the level of histological infection.

Neutrophils were scored on a previously used semi-quantitative scale of 0–3 with score 0 = no neutrophils; 1 = mild neutrophil aggregation; 2 = moderate neutrophil aggregation; 3 = severe neutrophil aggregation as indicated by Figure 1 in the uterine tissue and Figure 2 in the placental tissue (Tursi et al., 2010).

3.8. Statistical analysis

The time to abortion between groups was compared using Kaplan–Meier curves with the log rank test used to assess equality of survival distribution between the groups. Fisher’s exact test was used to compare the proportion of animals aborting, proportion of animals positive for *C. fetus* subsp. *venerealis* using culture or PCR and the proportion of animals with histological evidence of metritis or placentitis. Statistical analysis was performed using STATA (StataCorp LP, College Station, TX, USA).

4. Results

4.1. Baseline data

All animals were healthy, culture negative for *Campylobacter fetus* subsp. *venerealis* and were between 35 and 49 days pregnant when inoculated. Animals had a starting weight ranging from 862 to 1020 g (mean = 973 g; standard deviation = 52 g). Vaginal swabs taken from 20 guinea pigs prior to inoculation were all negative on culture of *C. fetus* subsp. *venerealis*.

4.2. Intra-peritoneal challenge

One hundred percent of the animals (excluding controls) in groups 1 and 2 (strain 258 10^9 CFU/ml) experienced abortion, compared with 0% in groups 3 (strain Q41 8.4×10^7 CFU/ml), 6 (strain 540 1.2×10^4 CFU/ml) and 7 (strain 540 IVA 5.4×10^7 CFU/ml) ($p = 0.03$). In groups 4 and 5 (strain 540), 75% of animals experienced abortion. No control animals aborted (Table 1).

Time to abortion was not statistically different between groups inoculated with the same strains at slightly different concentrations as seen in groups 1 and 2 with strain 258 at 1.0 and 1.5×10^9 CFU/ml ($p = 0.44$) and in groups 4 and 5 with strain 540 at 1.0×10^8 CFU/ml and 3.3×10^7 CFU/ml ($p = 0.62$) (Figure 3). Overall, the incidence of abortion differed between at least one of the groups 1–7 (group 1 vs 3, 6 and 7 $p = 0.0067$, group 2 vs 3, 6 and 7 $p = 0.0058$, group 5 vs 3, 6 and 7 $p = 0.0388$, group 3 vs 4 $p = 0.0401$) (Figure 3).

Culture isolations from peritoneum, uterus, placentas and amniotic fluid did not differ significantly between IP inoculated groups 1–5 ($p = 1.00$) (Figure 4). However, groups 1–5 isolations were significantly different from non-aborting groups 6 and 7 uterus ($p = 0.03$) and placenta culture compared with groups 1–5 (respectively; $p = 0.02, 0.01, < 0.001, < 0.001, < 0.001$). Amniotic fluid isolations from group 7 was significantly different compared with groups 4 and 5 (group 7 vs 1–6 respectively $p = 0.14, 1.0, 0.15, 0.007, 0.0005$ and 1.0). Isolations from foetal stomach fluid varied significantly between groups 2 and 4 ($p = 0.003$), groups 2 and 5 ($p = 0.024$) and between groups 4 and 5 and group 7 (group 4 vs 7 $p = 0.002$, group 5 vs 7, $p = 0.02$). Re-isolation of the bacteria from all inoculated dams is shown in Figure 4 and numbers of placental tissue, amniotic fluid and foetal stomach content compared per group is displayed in Table 1.

Biochemical and molecular profiling for each strain matched those of the inoculum. Comparative detection of the infectious organism through culture, histology or PCR showed culture to be the most consistent with re-isolation of the causative agent in every aborting dam as well as the non-aborting dams in group 3 (Figure 4). There was no difference in culture, histology or proportion of PCR positives at an animal level per group ($p = 1.00$) (Figure 5).

Histological evidence of infection correlated with abortion events, with non-aborting groups (group 6 and 7) showing no neutrophil aggregation (Table 1). Strain 540 at concentrations of 10^8 CFU/ml showed a higher level (average score = 2.4) of neutrophil cell infiltration than at 10^7 CFU/ml (average score = 1). A high level of suppurative infiltration was seen in most abortive dam tissues, with haemorrhage and vacuolation

visualized in only a few samples.

4.3. *Intra-vaginal challenge*

None of the four intra-vaginally inoculated animals nor the control aborted within the 12 day (268 h) timeframe, and all animals were culture and histology negative while one sample (dam spleen) was PCR positive for *C. fetus* subsp. *venerealis* (Figure 5).

Table 1. Summary of concentrations of three *Campylobacter fetus* subsp. *venerealis* strains used for inoculation of groups 1–7 of four guinea pigs and control group of seven guinea pigs (one per group) each via either intra-peritoneal (IP) or intra-vaginal (IVA) route as well as time to study end-point, interquartile range of time to and percentage of abortions. Number of samples from IP inoculated dams ($n = 4$ per group) which were culture positive for *Campylobacter fetus* subsp. *venerealis* in all foetuses (aborted and non-aborted) from groups 1 to 6. In the instance of abortions there was subsequent loss of samples due to fluid drying or dam eating all or part of placenta and foetus. Neutrophil aggregation scores for groups 1–7 as seen in uterus and placental tissues of inoculated dams. Foetal tissue samples (head, thorax, abdomen) showed no neutrophil infiltration suggestive of inflammatory response. Insufficient samples for an accurate representation of amniotic sac infection were collected due to loss during abortion and/or desiccation and are therefore not shown.

Group	Strain	Concentration	Inoculation route	End-point (h)	Percentage aborting	IQR time to abortion (h)	Culture placenta	Culture AF (n)	Culture FSC (n)	Neutrophil score uterus	Neutrophil score placenta
1	258	1.5×10^9 CFU/ml	IP	168	100%	51 (36–75) ^a	5/7	1/2	2/5	1.3	1.5
2	258	1.0×10^9 CFU/ml	IP	168	100%	67 (67–116) ^b	2/2	0/0	0/11	1.0	0.7
3	Q41	8.4×10^7 CFU/ml	IP	168	0%	N/A	12/12	2/8	0/4	0.5	0.8
4	540	1.0×10^8 CFU/ml	IP	168	75%	48 (19–72) ^c	15/15	9/14	8/14	2.8	2.0
5	540	3.3×10^7 CFU/ml	IP	288	75%	68 (68–140) ^d	15/15	4/4	6/15	1.0	1.0
6	540	1.2×10^4 CFU/ml	IP	Full-term	0%	N/A	0/0	0/0	0/0	0.0	N/A
7	540	5.4×10^7 CFU/ml	IVA	288	0%	N/A	0/12	0/12	0/12	0.0	0.0
Control ^e	N/A	Sterile broth	IP	168/288	0%	N/A	0/18	0/18	0/18	0.0	0.0

h, hours; IQR, interquartile range; N/A, not applicable; CFU/ml, colony forming units per millilitre; AF, amniotic fluid; FSC, foetal stomach contents; n , number. ^a Time to abortion significantly different from group 3, 6 and 7 ($p = 0.0067$). ^b Time to abortion significantly different from groups 3, 6 and 7 ($p = 0.0058$). ^c Time to abortion significantly different from groups 3, 6 and 7 ($p = 0.0401$). ^d Time to abortion significantly different from groups 3, 6 and 7 ($p = 0.0388$). ^e Control group consisted of 1 animal per group 1–7 inoculated with sterile broth via the same route and endpoint.

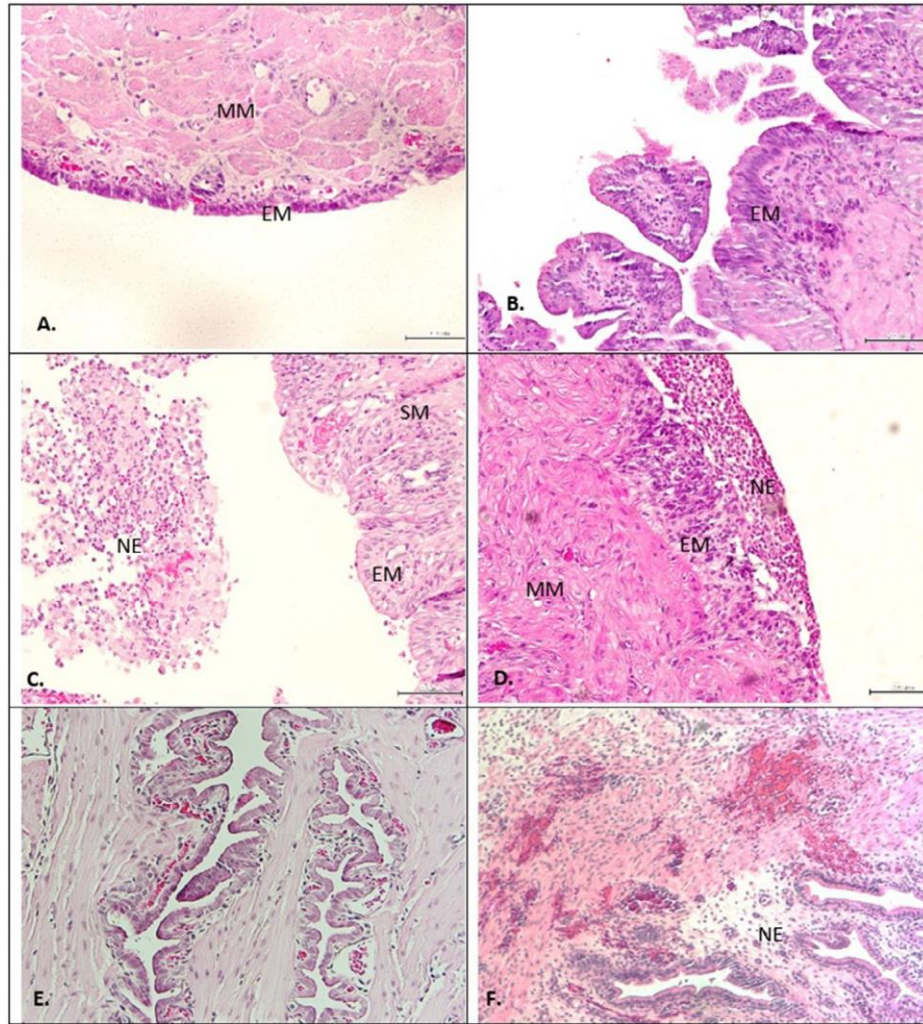


Fig. 1. Uterine photomicrographs from *Campylobacter fetus* subsp. *venerealis* inoculated guinea pigs showing the inflammatory scale used for grading of neutrophil cellular infiltration. H&E stain; bar = 200 μ m. (A) Scale 0; normal endometrium (EM) and myometrium (MM). (B) Scale 1; scattered neutrophils within the EM. (C) Scale 2; neutrophils within the submucosa (SM) and EM and neutrophil exudate (NE). (D) Scale 3; widespread accumulation of NE along the EM and scattered within the MM. (E) Non-infected control dam from group 1 showing EM tissue with no signs of inflammatory response (scale 0) $\times 400$. (F) Infected dam from group 1 showing hyper-cellularity with evidence of inflammatory response, most notably infiltration of neutrophils and congestion of tissue (scale 3) $\times 400$.

5. Discussion

This study found the pregnant guinea pig model to be a reliable and reproducible model for the study of *C. fetus* subsp. *venerealis* infection, strain virulence and dose dependency differences. The results of the study showed the reproducibility of the model with concentrations above 10^7 CFU/ml (strain 540 and strain 258). An increase in concentration of strain 540 by 1 log produced the same abortion rate (75%), with a decrease in the median abortion time by 20 h. Our results are consistent with a previous study where bovine strains of *C. jejuni*, *C. fetus venerealis* and *C. fetus* were tested at concentrations of 10^1 , 10^4 and 10^8 CFU/ml in pregnant guinea pigs (SultanDosa et al., 1983). Inoculated dams aborted at a rate of 60% ($n = 9$) for *C. fetus* (one strain), 13% ($n = 4$) for *C. jejuni* (two strains) and 73% ($n = 22$) for *C. fetus* subsp. *venerealis* (two strains) at all of the aforementioned doses within 21 days (SultanDosa et al., 1983). Abortions or abortive symptoms predominantly occurred within 2–17 days post-inoculation with an average of 7 days. The study also noted dose dependency, with the highest rate and shortest time to abortion produced at the highest dose amongst all strains used (SultanDosa et al., 1983).

In the present study, although none of the strain Q41 (ATCC19438) inoculated animals aborted, the bacterium was reisolated in all dams from a combination of four of the seven culture sites (Figure 4; Table 1). This strain appeared to be less virulent than the previous strain (258) within the timeframe (7 days) resulting in an increase in the study endpoint to 12 days to account for less virulent strains. Previous studies have used 2 (Burrough et al., 2011), 12 (Coid et al., 1987) and 21 days (Burrough et al., 2009) for *C. jejuni* and *C. coli*. However *C. fetus* subsp. *venerealis*, has been shown in cattle to require longer incubation times as seen in aborting heifers which are often affected in the third trimester of pregnancy (Hum et al., 2009). This was also seen in the initial *C. fetus* subsp. *venerealis* guinea pig study with up to 34 days required as the study end-point (Ristic and Morse, 1953). The shortest effective time-point was used in this study to avoid euthanizing fully developed foetuses in non-aborting dams.

In cattle, inflammation in the uterus is associated with infection as an indicator of campylobacteriosis associated endometritis (Clark, 1971). Placentitis has been described

as an infection mechanism of *C. fetus* subsp. *fetus* in ewes following bacteraemia, as well as in humans, as a mechanism of abortion due to orally transmitted *C. jejuni* and *C. coli* (Skirrow, 1994). Burrough et al. (2011) using a *C. jejuni* guinea pig model, showed that the organism had a high tropism for the placenta, suggesting the presence of a ‘chemoattractive placental factor’ which may account for the high level of placental isolations seen in their study.

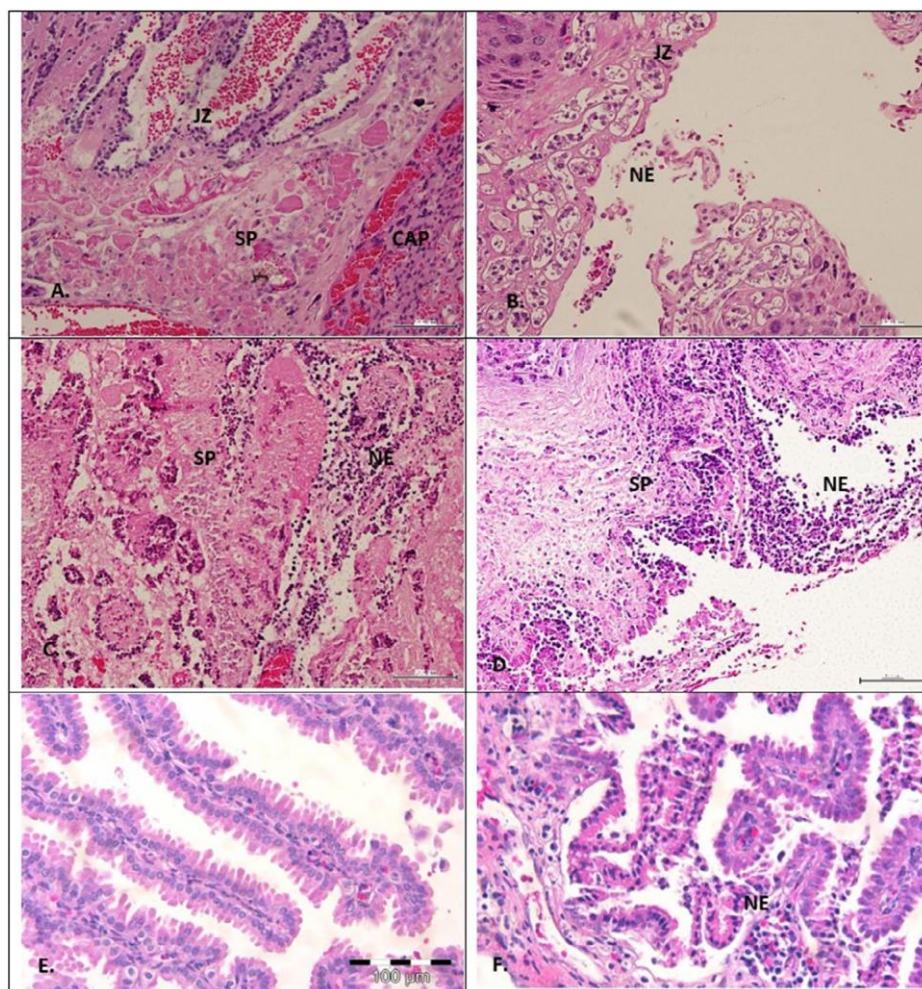


Fig. 2. Placental photomicrographs from *Campylobacter fetus* subsp. *venerealis* inoculated guinea pigs showing the inflammatory scale used for grading of neutrophil infiltration. H&E stain; bar = 200 µm. (A) Scale 0; normal placental tissue within the junctional zone (JZ), subplacenta (SP) and chorio-allantoic placenta (CAP). (B) Scale 1; scattered neutrophils within the JZ. (C) Scale 2; neutrophils within the SP and areas of neutrophil exudate (NE). (D) Scale 3; widespread accumulation of NE along and scattered within the SP. (E) Non-infected control dam from group 4 showing normal yolk sac placenta with no signs of inflammatory response (scale 0) ×400. (F) Infected dam

from group 4 yolk sac placenta showing congestion of blood vessels, necrosis of epithelial cells and moderate inflammatory cell infiltrate comprising neutrophils and lymphocytes (scale 3) $\times 400$.

In cattle the bacteria are transmitted venereally; however, studies have shown that fertilization itself is not affected by *C. fetus* subsp. *venerealis* but rather fertility decreased by a reduction in implantation (Bielanski et al., 1994; Skirrow, 1994; Wilkie and Winter, 1971). These studies suggest that infection of the guinea pigs at the time of fertilization was not necessary to produce a comparative model for bovine infection. We found that inoculation by the IVA route was ineffective in producing infection or abortion. It is possible that organisms failed to penetrate the cervix at the time of inoculation.

A more recent study into a virulence factor associated with *C. jejuni* infection, *luxS*, showed that it had the capability to affect motility, colonization and toxin production, and was directly linked to the bacteria's ability to produce abortion in guinea pig and chicken models (Plummer et al., 2012). While the *luxS* gene is present in *C. fetus* subsp. *venerealis* (Stynen et al., 2011) its involvement in bacterial pathogenicity is unknown. A disruption of *virD4* (an essential component of the bacterial Type IV secretion apparatus) in *C. fetus* subsp. *venerealis* showed a 50% reduction in cell invasion *in vitro* compared with the original wild type strain (Gorkiewicz et al., 2010). Such studies to date have been undertaken using *in vitro* culture invasion assays but could also incorporate the use of gene specific mutants to assist in the screening of potential virulence factors (Gorkiewicz et al., 2010; Kienesberger et al., 2007).

While the effect on fertilization could not be tested in this study as it was not possible to infect the guinea pigs at the time of mating, due to the similar end-point of this infection model and bovine pathogenesis – inflammation in the uterus and placenta causing early embryonic death and late abortions – this model is valid for use in studying bovine strain variations and infection mechanisms.

Culture has been, and still remains the gold standard for the diagnosis of BGC in infected cattle (World Organisation for Animal Health, 2012). Culture was reliable and consistent with high levels of re-isolation of bacteria from different sites in the animals. However,

culture sensitivity at an animal level was lower than that of PCR in this study, with one sample in a dam inoculated via the IVA route positive by PCR but negative by culture or histology. This could be due to the infection becoming systemic and clearing through the spleen leaving residual DNA. However, due to the low number of samples tested by PCR in this study, further investigation and validation would be necessary before its recommended use in further studies.

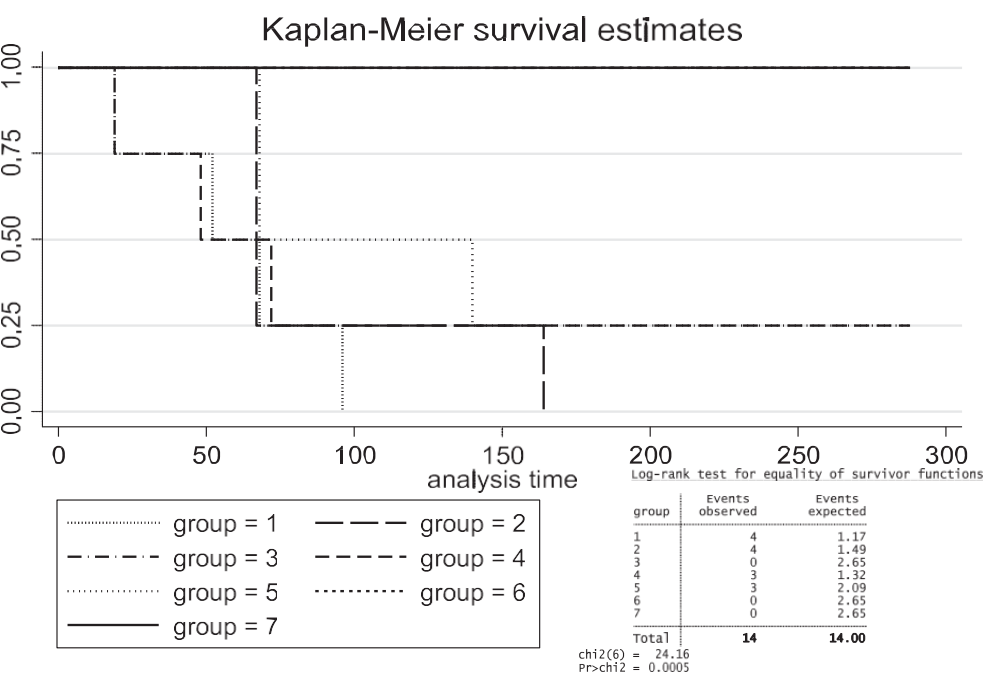


Fig. 3. Kaplan–Meier survival estimates of groups 1–7 showing abortion incidences to end-point with log rank for equality of survivor function, $\chi^2 = 24.16$ and $\text{Pr} > \chi^2 = 0.0005$.

This study has shown the difference between the ability of *C. fetus* subsp. *venerealis* strains to cause abortions at similar concentrations with strain Q41 (ATCC19438) having no effect while strains 258 and 540 had abortion rates of 100% and 75% respectively. Two studies using different strains (*C. fetus* and *C. jejuni*) at a concentration of 10^5 CFU/ml found that IP injection of pregnant guinea pigs produced 83% abortions with 21 days with *C. jejuni* and 100% abortions within 12 days with *C. fetus* (Burrough et al., 2009; Coid et al., 1987). However, in the current experiment the *C. fetus* subsp. *venerealis* strain 540 at 1.2×10^4 CFU/ml did not result in any abortions or show evidence of progression of infection.

Genomic profiling may identify differences which may explain these results and also provide insights into genes which may be associated with higher virulence. The *C. fetus* subsp. *venerealis* strains used in this study appear to require a higher infective dose to produce abortions when compared with both *C. fetus* and *C. jejuni* (Burrough et al., 2009; Coid et al., 1987). The differences between previous studies and the present study include animal model species, infective dose, time to abortion, geographical origin of the strains and strain pathogenicity.

A limitation is that only three different strains were tested, strain Q41 (ATCC19438) only once, as well as the limited doses tested. In contrast with other studies which used vaginal bleeding as the study endpoint, this study used abortion (defined as expulsion of one or more fetuses and/or placentas) as the endpoint (Burrough et al., 2011; Plummer et al., 2012). An important limitation on the interpretation of results occurred when samples were not readily collectable. In the instance of abortions, the dam would occasionally eat the placenta and/or part of the foetus and the amniotic sac become damaged and desiccated. With the occurrence of acute abortions, the presence of very small foetuses resulted in insignificant foetal stomach fluids and foetal spleen available for testing.

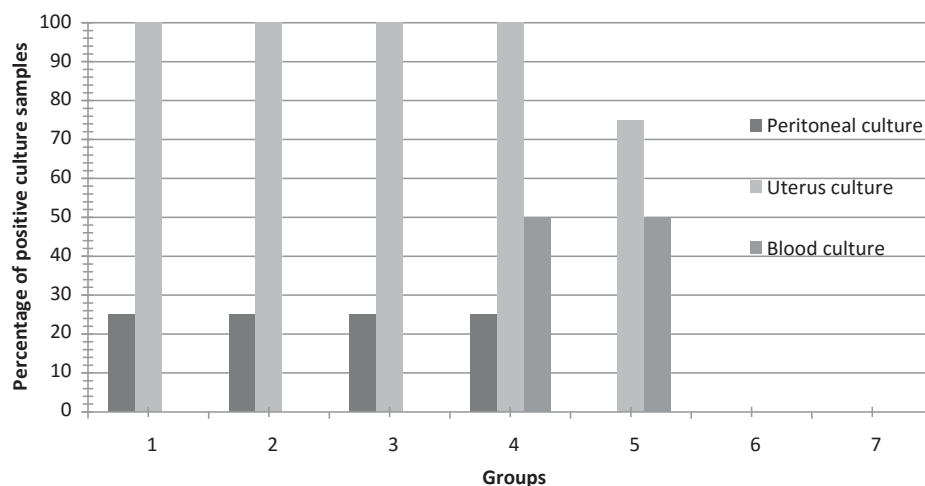


Fig. 4. Percentage of samples from each of the three anatomical sites of inoculated dams which were culture positive for *Campylobacter fetus* subsp. *venerealis* in aborting and non-aborting instances ($n = 4$) in groups 1–7 (see Table 1). No blood samples were collected in groups 1–3.

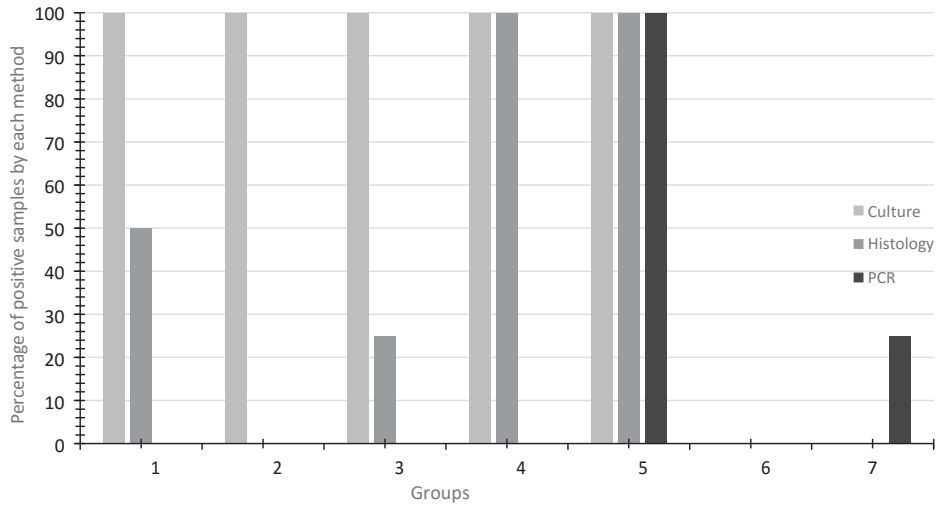


Fig. 5. Comparison of bacterial presence detected through culture, histology and PCR in inoculated animals ($n = 4$) of groups 1–7 (see Table 1). The criterion for a positive result was a minimum of one positive sample per animal for each test. Culture results are from isolations in peritoneum, uterus, placenta, blood, amniotic fluid or foetal stomach contents. Histological findings were associated with metritis or placentitis per animal and positive PCR results for uterus, spleen, serum or placenta for each inoculated animal. PCR was not done on samples in groups 1–4.

This study defined parameters for a route of infection, dose and study end-point to ensure the validity and reproducibility of a *C. fetus* subsp. *venerealis* infection model. It has also identified the most consistent sites for isolation of *C. fetus* subsp. *venerealis* and the most accurate method for evaluation of infection. The study has shown that the pregnant guinea pig model described here is promising for the assessment of further *C. fetus* subsp. *venerealis* strains. Defining the pathogenic and genomic variation of *C. fetus* subsp. *venerealis* strains may help ascertain the involvement of different bacterial virulence factors in BGC. This is a valuable model for the testing of different strains to improve the understanding of bovine genital campylobacteriosis.

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Chapter 7. Identification of Australian
***Campylobacter fetus* subspecies**
***venerealis* strain virulence variation in a**
guinea pig infection model

7.1 Manuscript Information

The following manuscript has been submitted to the journal Veterinary Research (VETR-D-15-00296).

The candidate, Ameera Koya, was primarily responsible for the study design and implementation as well as writing the manuscript. The co-authors: Rachel Allavena, Shirley Turner, Ristan Greer, Ala Lew-Tabor, John Power and Gry Boe-Hansen contributed to the manuscript on various aspects. The contributions of each co-author is listed on page viii of this thesis.

*Strain 600 used for strain variation assessment is B6.

Identification of Australian *Campylobacter fetus* subspecies *venerealis* strain virulence variation in
a guinea pig infection model

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Abstract

Bovine Genital Campylobacteriosis (BGC), caused by *Campylobacter fetus* subspecies *venerealis* is associated with cattle production losses due to herd infertility and sporadic abortions. The aim of this study was to compare abortifacient properties between *C. fetus* subsp. *venerealis* strains in a pregnant guinea pig model. Two experiments were conducted; 1) Determining a protective dose of an experimental bivalent vaccine against the bacteria, and, 2) Using this dose as a parameter for testing the virulence of 4 strains. In Experiment 1 the most protective dose was determined by testing the effects of four different vaccine doses (1:0, 1:2.5, 1:10, and 1:25) against a *C. fetus* subsp. *venerealis* strain (540) at a concentration shown to cause 75% abortions previously. The lowest number of abortions was detected in the 1:2.5 dose group and was used in Experiment 2, to assess the virulence differences of four Australian *C. fetus* subsp. *venerealis* strains. Four groups of 10 pregnant guinea pigs with four vaccinated twice, five weeks apart prior to mating and 6 non-vaccinated were challenged intra-peritoneally at week five of gestation with one of four different strains (76223, 924, 635 and 600) at a concentration of 10^7 CFU/ml. Tissues from dams and foetuses were examined by culture, histology and PCR. Proportions were compared using Fisher's Exact test. Strain 76223 resulted in 8/10 abortions (6/6 non-vaccinated, 2/4 vaccinated), strain 924; 2/10 (2/6 non-vaccinated, 0/4 vaccinated), strain 635; 1/10 (1/6 non-vaccinated, 0/4 vaccinated), and strain 600; 2/10 (1/6 non-vaccinated, 1/4 vaccinated), within 12 days ($p=0.0006$). *C. fetus* subsp. *venerealis* was reisolated most commonly from uterus horns in both experiments, with unsuccessful reisolation in 11 vaccinated and 7 non-vaccinated animals. Virulence variation was evident with differences between the four strains in abortion rates, culture reisolations, histopathological inflammation, and PCR results with strain 76223 the most virulent. Identification of *C. fetus* subsp. *venerealis* strain pathogenic differences, virulence markers and vaccine efficacy studies could be explored using this small animal model.

Keywords: Campylobacteriosis, vaccine, virulence, guinea pig, cattle

Introduction

Bovine Genital Campylobacteriosis (BGC), caused by *C. fetus* subspecies *venerealis* is present in many extensive cattle farming countries, including Australia (World Organisation for Animal Health 2012). It causes significant economic losses due to reduced calving rates, trade restrictions and culling of infected animals (Clark 1971). The motile, microaerophilic bacteria, are often carried asymptomatically by bulls and transmitted venereally. Not all infections appear to be symptomatic, with intermittent vaginal secretions and oestrus cycle changes the only indicators in some infected heifers (Hum, Hornitzky et al. 2009). In these heifers, the bacteria was found to most commonly colonise the uterus and occasionally foetuses, causing sporadic abortions and infrequent infertility (Butzler 1984). The OIE (World Organisation for Animal Health) has classified BGC as a notifiable cattle disease, with most countries requiring certification of negative status for export of semen (World Organisation for Animal Health 2012).

Preventive measures include artificial insemination with certified negative semen or mating with young, virgin bulls, both of which are costly and impractical for extensive farms. Prevention is of higher importance in Australian farms where natural service in large herds is the normal breeding protocol compared to parts of Europe that have less extensive production systems allowing different preventative measures to be taken (Bondurant 2005). Other farming practices for the treatment of infected cattle include the use of dihydrostreptomycin for washing out the penile sheaths as well as subcutaneous streptomycin injections to treat infected bulls, however, antibiotic resistance can reduce the effectiveness of these strategies (Eaglesome and Garcia 1992). Vaccines for the prevention of BGC are available through different manufacturers in countries possessing large cattle production economies, to be administered as a preventative practice to herds annually.

In order to verify the best strains to use in a killed bacterial vaccine against *C. fetus* subsp. *venerealis*, the pathology associated with these strains needs to be assessed to identify strains possessing a wide range of virulence factors and inducing a suitable protective immune response. There is currently limited knowledge on the variation of virulence between strains present in Australian herds, which is an important first step in determining strains suitable for vaccine development. *In vitro* tissue models testing mutated bacterial strains (Kienesberger, Gorkiewicz et al. 2007, Gorkiewicz, Kienesberger et al. 2010) or small animal models detecting biological responses could be used in this process (Ristic and Morse 1953, Coid, O'Sullivan et al. 1987, Burrough, Sahin et al. 2009, Burrough 2011).

Guinea pigs have been used in the study of *Campylobacter* species with great success (Ristic and Morse 1953, Bryner 1976, SultanDosa, Bryner et al. 1983, Coid, O'Sullivan et al. 1987, Burrough, Sahin et al. 2009, Burrough 2011, Burrough, Sahin et al. 2011). They are susceptible to teratogenic stimulants, and sensitive to *Campylobacter* infections, as well as effective vaccine responders (Bryner, Foley et al. 1978, Bryner, Foley et al. 1979, Bryner, Firehammer et al. 1988, Burrough 2011). An initial study by Ristic and Morse was successful in producing a model for the study of *C. fetus* subsp. *venerealis* infection in pregnant guinea pigs (Ristic and Morse 1953). A previous study by our group into the use of a refined guinea pig model for the assessment of Australian *C. fetus* subsp. *venerealis* strains was effective in defining parameters and histological examination markers for identifying infection (Koya, de Wet et al. 2015).

The application of a small animal model for the assessment of a range of Australian *C. fetus* subsp. *venerealis* strains would allow testing of a larger number of strains than may be feasible in cattle studies. This would provide an opportunity to explore the level of *C. fetus* subsp. *venerealis* strain variation and, thereby, potentially identify new strains for inclusion in a an improved vaccine (initially developed in the 1970's (Clark, Dufty et al. 1977)). It would also allow analysis of the vaccine against a number of strains to determine efficacy at a lower cost compared with cattle trials.

This study aimed to identify potential Australian *C. fetus* subsp. *venerealis* strain differences based on physiological response in a previously validated pregnant guinea pig model in the form of abortion, with vaccine protection as a virulence indicator, as well as identifying infection mechanisms through bacterial culture, DNA detection by PCR and indication of inflammatory response by histological identification of pathology markers.

Aims:

Experiment 1

The aim of Experiment 1 was to determine the dose of an experimental bivalent vaccine containing whole-killed cells of *C. fetus* subsp. *venerealis* biovar *venerealis* and *C. fetus* subsp. *venerealis* biovar *intermedius* which would induce protection against *C. fetus* subsp. *venerealis* strain 540. This strain has previously been shown to cause abortion in guinea pigs.

Experiment 2

The aim of Experiment 2 was to determine the virulence variation of four strains, using vaccination as a parameter.

Hypotheses

It was hypothesized that the number of abortive incidences as well as the level of protection offered by the vaccine would vary according to the inherent virulence of the strain.

Material and Methods

Study Design

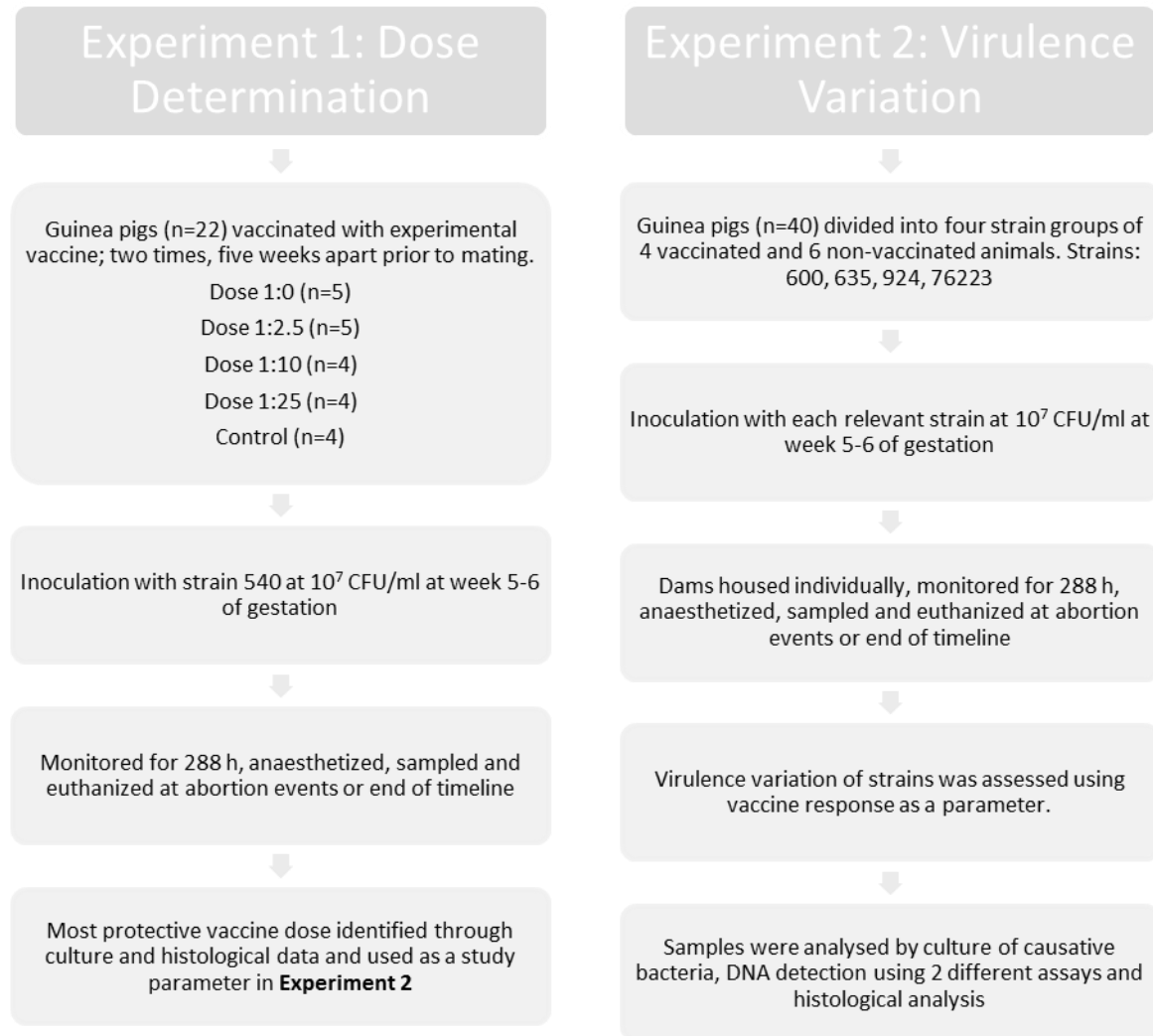


Figure 1. Schematic of study design with explanation of Experiments 1 (dose determination) and 2 (virulence variation) showing number of animals used in each study per dose/strain, inoculation, monitoring and assessment using relevant techniques.

Experiment 1: Protective Dose Determination

This was a parallel arm controlled trial where animals were allocated to five groups, four treatment groups (n=5 in two groups and n=4 in two groups) each treated with a different vaccine dose, and one control group (Figure 1).

Groups of female guinea pigs were vaccinated with experimental vaccine twice, five weeks apart, prior to mating. Groups received experimental vaccine at dilutions of 1:0, 1:2.5, 1:10, 1:25, with the control group receiving sterile mineral oil adjuvant used in the vaccine. At 5-6 weeks gestation they were inoculated with *C. fetus* subsp. *venerealis* strain 540 at a dose of 10^7 CFU/ml. Animals were then monitored for up to 12 days (288 h). At the occurrence of an abortion, or at the end of the timeline where no abortions occurred, animals were anaesthetized, sampled and euthanized.

Experiment 2: Strain Virulence Variation

From Experiment 1, the most protective dose as determined from abortion events and identification of infection through culture, PCR and histopathology, was used as an indicator of strain virulence for Experiment 2.

Four groups of 10 guinea pigs (4 vaccinated and 6 non-vaccinated in each group) were inoculated with one of 4 strains (600, 635, 924, and 76223) and monitored for abortion for up to 12 days (288 h), at which time remaining animals were sampled and euthanized (Figure 1).

Ethics

This study was approved by the University of Queensland Animal Ethics Committee (SVS070/10).

Animals

A total of 62 tri-coloured female guinea pigs (*Cavia porcellus*) were obtained from the University of Queensland's guinea pig breeding facility. Guinea pigs were housed in floor pens with wood shavings with access to vitamin C supplemented water (1 g/l) and Barastoc guinea pig pellets (Ridley AgriProducts, Melbourne, Australia) *ad libitum* as well as fresh vegetables daily.

For both experiments, guinea pigs were vaccinated as required, and randomly housed in groups of 6-8 females with one male for mating purposes.

Vaccination

Five bivalent experimental vaccines (Zoetis, Melbourne, Australia) were formulated to contain mineral oil adjuvant and a range of total antigen concentrations consisting of 8.0, 3.2, 0.8, 0.32, and 0 grams dry mass of killed cells per Litre. These experimental formulations corresponded to the respective dilution series described as 1:0, 1:2.5, 1:10, and 1:25 dilution with the vaccine formulated without antigen serving as an adjuvant-only control. All vaccines were tested for sterility and shown to be sterile prior to release for further experimentation. A 23G needle was used to inject 1.0 ml of the relevant vaccine subcutaneously on the left of the scruff after sterilising with iodine, with a 0.5 ml booster dose five weeks later. All guinea pigs were placed with a male two weeks post booster vaccination. At each vaccination time-point the vaccine sterility was checked by placing a drop onto a sheep blood agar (SBA) plate (Oxoid Australia, Thermofisher Scientific, Waltham, MA, USA) streaking and incubating aerobically at 37°C for 7 days.

The vaccine dose selected from Experiment 1 was delivered by the same method to 16 guinea pigs. All vaccinated and non-vaccinated animals were placed with a male two weeks after administering the booster vaccine.

Pregnancy Determination

During the mating period, the guinea pigs were oestrus-detected daily through two oestrus cycles and monitored by transabdominal ultrasound when oestrus negative for 21 consecutive days as done in a previous study (Koya, de Wet et al. 2015). The presence of foetuses with heartbeats detected confirmed pregnancy and gestation backdated to oestrus. Animals were inoculated as they reached week 5-6 of pregnancy.

Inoculum Preparation

Experiment 1

A previously used strain of *C. fetus* subsp. *venerealis* (strain 540) obtained from a bull prepuce collected from an abattoir sampling (Indjein 2013) was used. This strain previously produced abortions in the model and had been used to determine the infective dose (Koya, de Wet et al. 2015).

Experiment 2

The strains used in Experiment 2; strain 600, 635, 924 and 76223, were isolated from cattle in Australia with biochemical testing matching those outlined by the OIE and molecular profiles

composed of PCR results (Hum, Quinn et al. 1997, McMillen, Fordyce et al. 2006, Abril, Vilei et al. 2007) as well as virulence markers (VirB10, VirD4, VirB8/9 and VirB11) (Gorkiewicz, Kienesberger et al. 2010, Indjein 2013). Strains 635 and 924 were collected as part of a bull prepuce survey and isolated from abattoir material (Indjein 2013) while strain 600 was the *C. fetus* subsp. *venerealis* biovar *venerealis* strain used in the experimental vaccine and strain 76223 was isolated from placental tissue of a diagnostic case of abortion in a cow in Queensland, Australia. These strains were selected based on their viability after resuscitation as measured in a previous study (Koya, de Wet et al. 2015) and furthermore for strain 600, its inclusion in the vaccine; for strain 635, its biochemical and molecular profile matching that of strain 600; and for strain 924, resistance to tetracycline and streptomycin (Table 1 and 2).

Table 1. Molecular profile of *C. fetus* subsp. *venerealis* strains used in this study including *CstA/parA* conventional PCR, Abril and McMillen RT PCR assays and pathogenicity island gene PCR assays.

Strain	Conventional and RT PCR assays			Pathogenicity island genes PCR assays			
	<i>CstA/parA</i>	Abril	McMillen	VirB8/9	VirB10	VirB11	VirD4
540	+	+	+	-	-	-	-
600	+	+	+	+	+	+	+
635	+	+	+	+	+	+	+
924	+	+	+	-	-	-	-
76223	+	+	+	-	+	-	+

Table 2. Biochemical profile of *C. fetus* subsp. *venerealis* strains used in the study as identified by OIE stipulated characteristics.

Strain	Ox	Cat	25°C	42°C	TSI	H ₂ S	1% Gly	NA	K	T	S
540	+	+	+	-	-	-	-	R	S	S	S
600	+	+	+	-	-	-	-	R	S	S	S
635	+	+	+	-	-	-	-	R	S	S	S
924	+	+	+	-	-	+	-	R	S	R	R
76223	+	+	+	-	-	-	-	R	S	S	S

*Ox – oxidase; Cat – catalase; 25°C/ 42°C – growth at specified temperature; TSI – H₂S production on TSI slope; H₂S – H₂S production in 0.02% cysteine broth using lead acetate paper; 1% Gly – growth in 1% glycine medium; NA – sensitivity to nalidixic acid (30µg); K – sensitivity to cephalothin (30µg); T – sensitivity to tetracycline (30µg); S – sensitivity to streptomycin (10µg)

Bacterial concentrations for both experiments were used based on growth at 72 h which was 10^7 CFU/ml as determined from plate and Helber chamber counts (Meynell and Meynell 1970). Each inoculum was plated out on the day of inoculation and incubated aerobically and microaerobically at 37°C to ensure purity of the broth.

Inoculation

For both experiments all dams were inoculated aseptically via the intra-peritoneal route with 1 ml of the relevant strain, left of the midline as previously described (Koya, de Wet et al. 2015).

Animal Monitoring

After inoculation, the animals were placed into individual pens and were assessed twice daily (early morning and evening) with an abdominal ultrasound to identify foetal number and health through movement and heartbeat. A physical exam was also performed detecting signs of systemic illness, including increase in temperature or respiration, vaginal opening, bleeding, or abortion in the dam. Abortion was defined as the expulsion of one or more foetuses or placentas, therefore, animals displaying vaginal bleeding without foetal death as identified from an ultrasound or expulsion of a foetus were closely observed but not considered to be aborting.

Study Parameters

Time from inoculation to abortion or time to euthanasia if no abortion had occurred by the end of the study period (288 h) was the main study parameter of both experiments. At the occurrence of an abortion event or 288 h had no abortions occurred, the dam was anaesthetized, with tissues sampled for bacterial culture (uterus horns, placenta, amniotic fluid, foetal stomach contents and blood), PCR (dam and foetal spleen, placenta and uterus) and histology (placenta and uterus) as previously described (Koya, de Wet et al. 2015).

Secondary outcomes for both experiments were culture of the strain from the dam and foetuses. For Experiment 2, evidence of the bacteria in tissues was determined using the *CstA/parA* duplex conventional PCR specific for *C. fetus* subsp. *venerealis* (Hum, Quinn et al. 1997) and the NahE qPCR specific for *C. fetus* concurrently (van der Graaf-van Bloois, van Bergen et al. 2013).

Histopathology

Tissues were harvested and fixed in an excess of 10% neutral buffered formalin for a minimum of one week prior to routine processing and paraffin-embedding. Sections were cut at 4 microns and

stained by routine methods with Haematoxylin and Eosin. For Experiment 1 tissues were graded using an empirical scale; 1- mild, 2- moderate, 3-severe as previously published (Koya, de Wet et al. 2015). For Experiment 2 tissues were graded using an expanded empirical scale; 1- minimal, 2- mild, 3- moderate, 4-marked, 5- severe; allowing for subtle discrimination in the more severe pathology seen when comparing multiple strains.

When expelled from the dam a separate aborted placenta (AP) was evaluated consisting of the umbilical cord remnants and amnion if present, the discoid placenta consisting of the parietal yolk sac placenta, interlobium, labyrinth, subplacenta and junctional zone (if present). The myometrium in aborting dams was analysed separately by examination of the separate placental site and associated myometrium. In non-aborting animals the entire placental unit (PU) was evaluated attached to the myometrium at the placental site. Rarely some tissues from aborting dams were unavailable for examination due to consumption by the dam. Each anatomic site was graded and analysed separately by histopathological examination. All placentae and placental sites were evaluated from every dam; however in the case of aborted placentae it was not possible to directly match aborted placenta to a specific placental site.

Foetuses were not routinely examined in this study as no histopathological abnormalities in any foetus were detected in work previously published (Koya, de Wet et al. 2015).

Statistical Analysis

The time to abortion between vaccine dose groups (Experiment 1) and between strain variation groups (Experiment 2) was compared using Kaplan-Meier curves and log rank test. Time to abortion was reported as the median, inter-quartile range (IQR). The proportion of animals aborting, and/or positive for *C. fetus* subsp. *venerealis* on culture or PCR, and the proportion of animals with histological evidence of metritis, placentitis or amnionitis, was compared using a Chi-Square or Fisher's exact test. Statistical analysis was performed using STATA (StataCorp LP, Texas, USA).

Results

Experiment 1: Vaccine dose determination

Abortion

There was no statistical difference in the number of abortions between the vaccine dose groups (dose 1:0 vs. 1:2.5 $p=1.00$, dose 1:0 vs. 1:10, 1:25 $p=0.52$, dose 1:2.5 vs. 1:10, 1:25 $p=0.21$) with long-rank test for survival not significant ($\chi^2 = 7.3$ $p=0.12$). Dose 1:2.5 group had the lowest abortion rate, with only 1 incidence. An average of 2.67 abortions occurred in the remaining three dose groups with 3 abortion incidences in the control group (Table 3).

Table 3. Guinea pigs in Experiment 1 vaccinated with five different doses of an experimental vaccine against *C. fetus* subsp. *venerealis*; (1:0, 1:2.5, 1:10, 1:25 and control adjuvant) showing total number of animals, aborting dams per group, statistical expectation of abortion event as calculated from log-rank test for equality of survivor functions ($\chi^2(4) = 7.3$; $\text{Pr}>\chi^2 = 0.1209$) and median time to abortions in hours (h) with interquartile (IQR) time variations within the 288 h time period.

Group	Total	Aborted	Events Expected	Median, IQR time to abortion (h)
1:0	5	2	3.67	249 (211-286)
1:2.5	5	1	3.33	26
1:10	4	3	1.14	19 (19-41)
1:25	4	3	2.27	164 (115-212)
Control	4	3	1.59	65 (60-73)

Culture

Culture of the inoculum bacteria was most common from the uterus in both control and vaccinated dams, 75% and 78% respectively (Figure 2). Isolations from the placenta were possible in 78% of available placental tissues from the control group, and 65% of vaccinated dam placentas. Vaccine dose 1:2.5 which produced the lowest number of abortions, showed recovery of the bacteria from 80% of uteri and 53% of placental tissue samples (Figure 3), which was not significantly different to control animals ($p=1.00$).

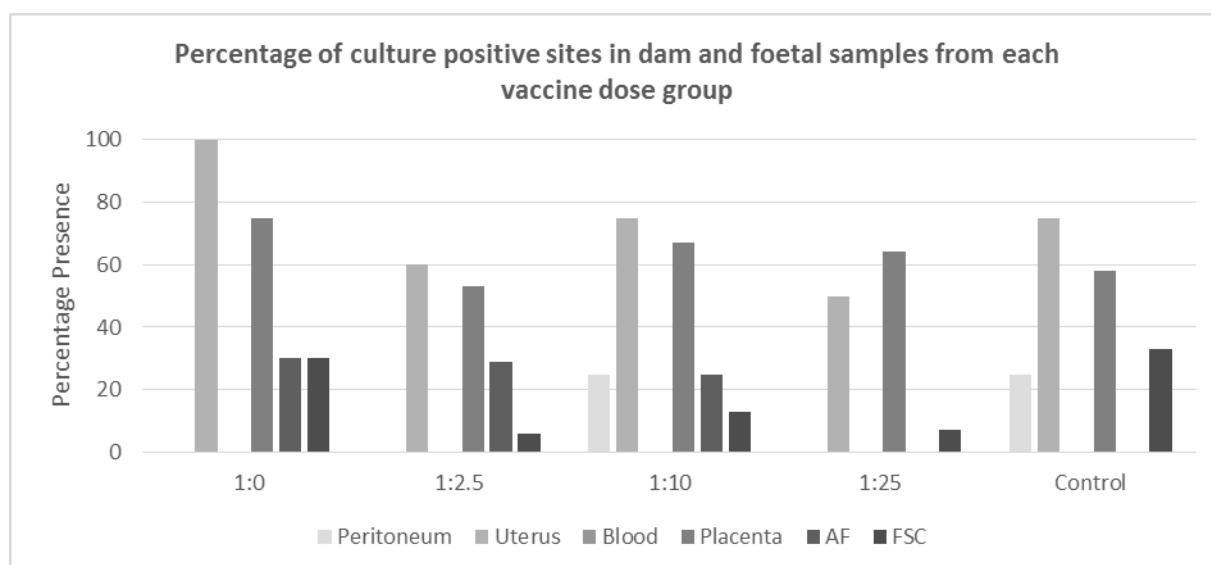


Figure 2. Percentage of inoculated dams and foetal samples per vaccine dose group (Experiment 1) which were culture positive for *C. fetus* subsp. *venerealis* in 1 of 6 areas (dam: peritoneum, uterus and blood; foetus: placenta, amniotic fluid (AF), foetal stomach contents (FSC)). The control group was sham injected and inoculated with *C. fetus* subsp. *venerealis* along with the other four dose groups; experimental vaccine at dilutions of 1:0, 1:2.5, 1:10, 1:25, with the control group receiving sterile mineral oil adjuvant used in the vaccine.

Histopathology

Polymorphonuclear (PMN) or neutrophilic infiltration causing fibrinosuppurative placentitis was seen across all groups, most commonly at the junctional zone infiltrating into the endometrium and myometrium (Figure 3). The average score graded for histological inflammation seen in control animals (n=4) was 1.33 in uterine tissue and 0.78 in the placental unit. Overall, vaccinated dams had a score of 1.36 and 0.82 in uterine and placental tissues respectively. Vaccine dose 1:2.5 produced inflammation scored as 0.80 for uterine tissue and 0.71 in placental tissue.

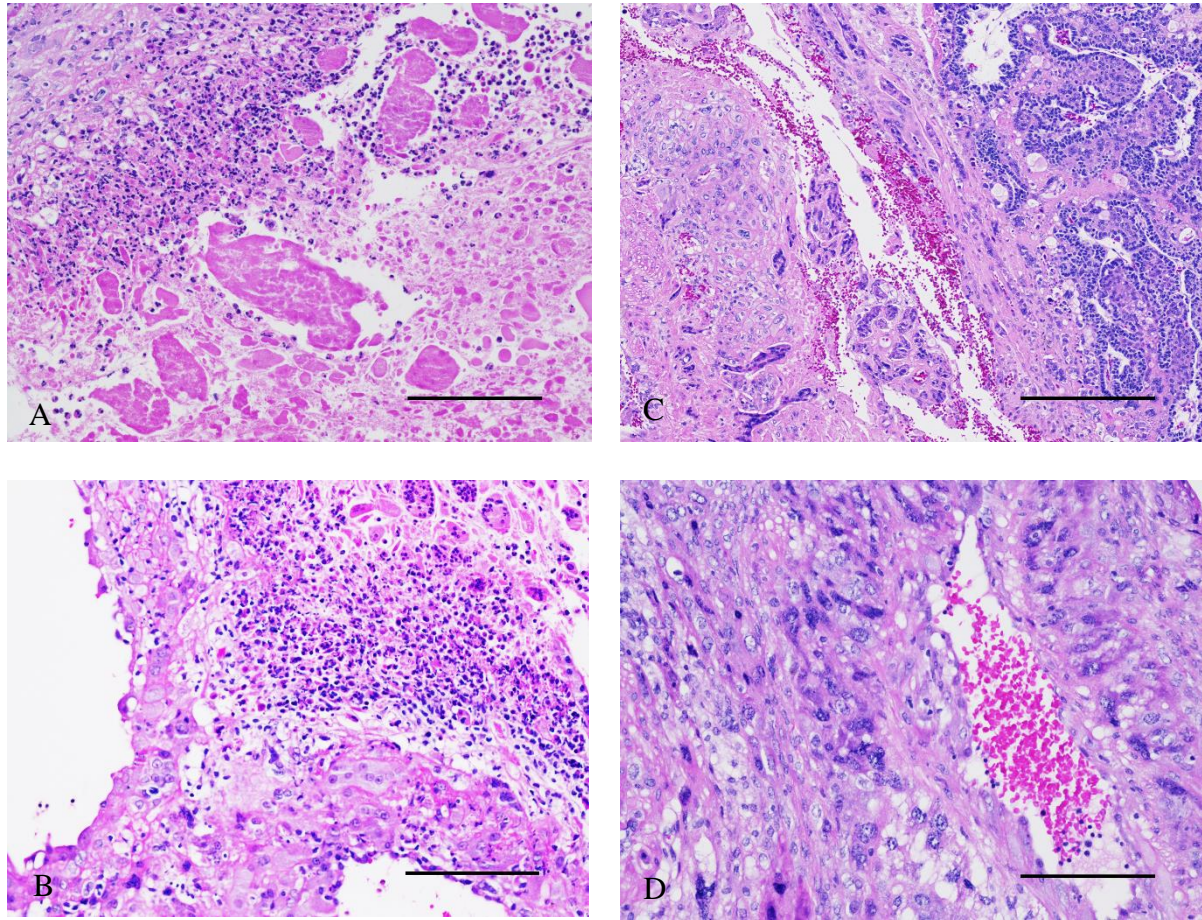


Figure 3. Photomicrographs of uterine and placental tissue sections from Experiment 1 guinea pigs inoculated with strain 540 of *C. fetus* subsp. *venerealis* in control adjuvant vaccinated dams and dams vaccinated with dose 1:2.5 of experimental vaccine. A – control vaccinated dam showing neutrophilic exudate and infiltration in the junctional zone of grade 2. H&E stain; bar = 100 μ m. B – control vaccinated dam uterine tissue showing diffuse neutrophils in endometrium, grade 1. H&E stain; bar = 100 μ m. C – dose 1:2.5 vaccinated dam showing no histological changes in the junctional zone, grade 0. H&E stain; bar = 200 μ m. D – dose 1:2.5 vaccinated dam displaying normal endometrial tissue with trophoblasts, grade 0. H&E stain; bar = 100 μ m.

Experiment 2: Strain Virulence Variation

Abortion

Occurrence of abortion varied according to strain with strain 76223 significantly different to all others (vs. 924 and 600 $p=0.02$, vs. 635 $p=0.0055$) while strain 600, 635 and 924 were not significantly different from each other ($p=1.00$). Log-rank test for survival over the four strains was significantly different ($\chi^2 = 17.2$ $p=0.0006$). Strain 76223 produced 100% abortions in non-vaccinated guinea pigs while others (strain 600 and 635) only 17% (1/6) (Figure 4, Table 4). In the vaccinated animals there were no abortions in two groups (strain 635 and 924), however, the two remaining groups had either 25% or 50% abortions within 82 h of inoculation (strain 600 and 76223).

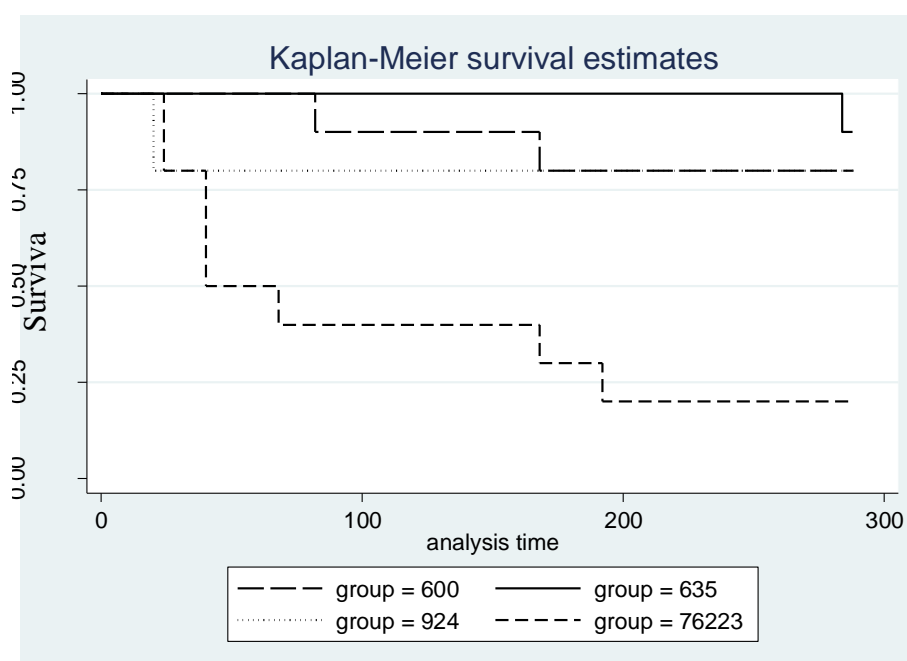


Figure 4. Kaplan-Meier survival curve showing 13 abortion incidences for Experiment 2 with four groups of 10 guinea pigs (4 vaccinated, 6 non-vaccinated) inoculated with different strains of *C. fetus* subsp. *venerealis* until endpoint (288 h). Chi-squared = 17.27; p-value = 0.0006.

Table 4. Percentage and interquartile range time to abortion for non-vaccinated (n=6) and vaccinated (n=4) guinea pigs inoculated with four different strains of *C. fetus* subsp. *venerealis* (strain 600, 635, 924 and 76223) in Experiment 2, within 288 h as well as number of animals per strain returning a positive culture or PCR result from any tested site.

Strain	Non-vaccinated animals (n=6)				Vaccinated animals (n=4)			
	Aborted (%)	Median, IQR time to abortion (h)	PCR positive *	Culture positive	Aborted (%)	Median, IQR time to abortion (h)	PCR positive *	Culture positive
600	17	168	1/6	1/6	25	82	1/4	1/4
635	17	284	6/6	6/6	0	N/A	1/4	1/4
924	33	20	2/6	4/6	0	N/A	0/4	0/4
76223	100	54 (40-168)	4/6	6/6	50	32 (24-40)	2/4	2/4

*positive on either Hum or NahE PCR; h, hours; IQR, interquartile range; n, number

Culture

All aborting dams were positive for reculture of the inoculated bacteria, as well as two non-aborting dams from strain 924 and 6 non-aborting dams from strain 635. Strain 600 showed no presence of the bacteria by culture in the remaining 8 non-aborting dams. Culture appeared more sensitive than molecular testing (Table 4), and was supported by histopathological findings in all culture positive animals.

Molecular Testing

Bacterial DNA detection in samples by NahE qPCR was most common overall from pooled placental tissue in non-vaccinated animals; 10/24 and vaccinated animals; 4/16. Five of the 24 non-vaccinated animals had detectable levels of DNA in uterine tissue with none of the vaccinated animals producing a positive result. There was detection of bacterial DNA in only one pooled foetal spleen sample from strain 76223 (vaccinated animal). Results from the *CstA/parA* duplex PCR produced fewer positives, with a large number of samples producing only one of the two bands (142 and 764 bp) expected for a positive result. In non-vaccinated animals, 11 samples produced true positive results (both 142 and 764 bp), while in the vaccinated animals only two samples from a single animal produced a true positive result. Of the non-vaccinated animals, all groups produced PCR positive results across either/or of the two PCR test methodologies (17% - 100% of animals) while strain 600 only had a single positive animal by the NahE PCR and was negative by the Hum PCR across all animals (Table 4).

Histopathology

C. fetus subsp. *venerealis* induced variably severe fibrinosuppurative placentitis, endometritis, necrosis and neutrophilic vasculitis, depending on strain and vaccination status (Figure 5, Figure 6). Changes were most severe and consistent within the junctional zone, but in virulent and highly abortive strains like 76233 neutrophilic inflammation could be seen in most compartments of the placental unit (PU).

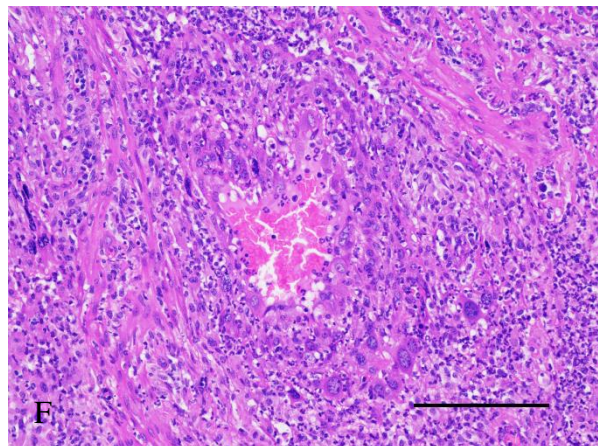
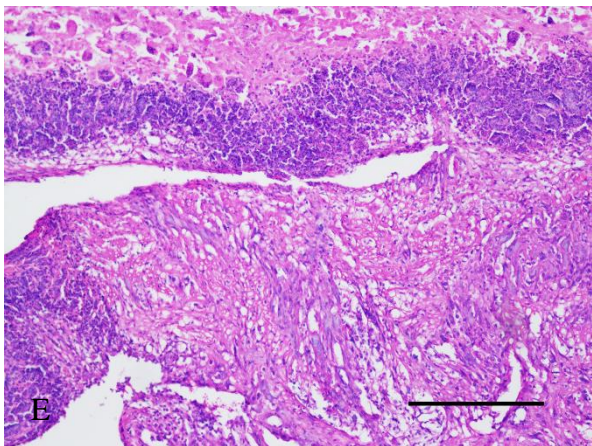
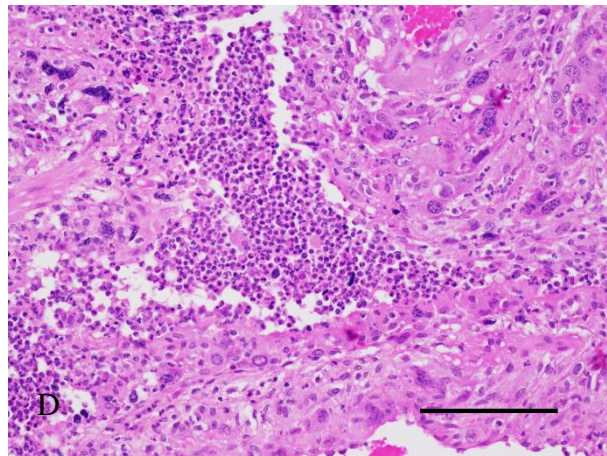
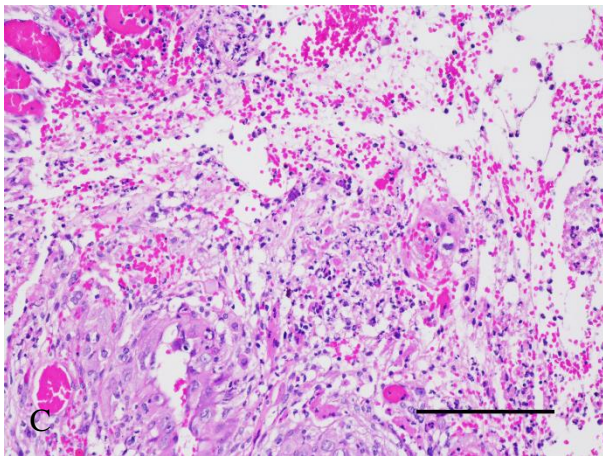
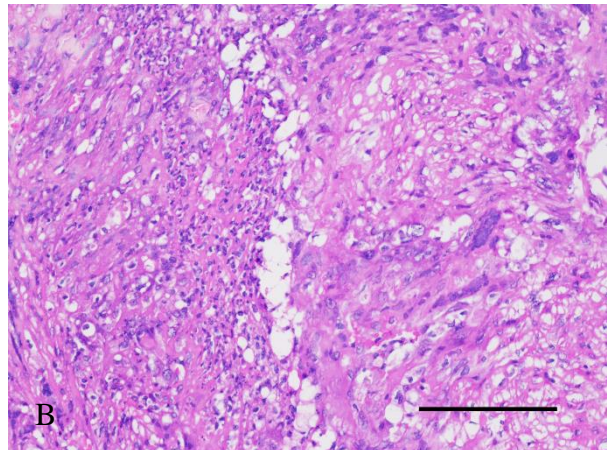
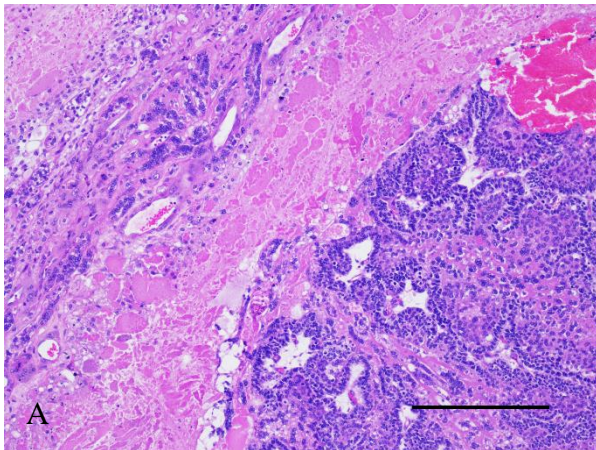


Figure 5. Photomicrographs of placental and uterine tissue from Experiment 2 showing examples of empirical grading scale (0– no pathological change, 1- minimal, 2- mild, 3- moderate, 4– marked, and 5- severe). A – grade 0; Normal subplacenta and junctional zone. H&E stain; 200 µm. B – grade 1; Myometrium subjacent to the junctional zone showing neutrophils and minimal debris. H&E stain; 100 µm. C – grade 2; Junctional zone with mild neutrophilic inflammation, congested vessels, fibrin and haemorrhage. H&E stain; 100 µm. D – grade 3; Myometrial tissue with trophoblastic invasion with aggregation of neutrophils causing moderate suppurative metritis. H&E stain; 100 µm. E – grade 4; Junctional zone with extensive degenerate neutrophilic exudate, fibrin, debris and necrosis. H&E stain; 200 µm. F – grade 5; Myometrium with multifocal neutrophils, debris and vascular inflammation. Invading trophoblasts are present. H&E stain; 100 µm.

Strain 76223

In non-vaccinated animals of strain 76223 pathologic lesions were most severe in the junctional zone though most compartments were variably affected across infected animals. The junctional zone exhibited moderate to severe fibrinosuppurative inflammation and neutrophilic and sometimes necrotising vasculitis with haemorrhage in highly affected animals. Necrosis was characterised by aggregation of sloughed and degenerate cells with nuclear pyknosis and karyorrhexis. Severely affected animals also additionally showed areas of necrosis, fibrin and suppurative vasculitis in the subplacental zone. Less severely affected placentae exhibited mild to moderate neutrophilic inflammation and haemorrhage in the junctional zone. Minimal to mild neutrophilic margination within the vasculature was seen in a majority of aborted placenta (AP) and PU examined in the parietal yolk sac placenta but was inconsistent in the visceral yolk sac placenta. The labyrinth and interlobium were infrequently affected with minimal or mild fibrinosuppurative inflammation and rare necrosis or vasculitis. The umbilical cord inconsistently showed focal or multifocal haemorrhage within Wharton's jelly, and very rarely neutrophilic infiltrates or vasculitis. Minimal to moderate neutrophilic exudates, sometimes with fibrin and haemorrhage were noted in the amniotic cavity more consistently in severely affected placentae. In dam tissues placental sites or placental units showed mild to moderate fibrinosuppurative endometritis which typically didn't extend into the underlying myometrium.

In vaccinated animals challenged with strain 76223 complete and partial abortions were noted in two animals (V25 and V33) which showed similar but slightly milder lesions to non-vaccinated aborting animals challenged with strain 76223. This included minimal to marked fibrinosuppurative

and haemorrhagic inflammation in the junctional zone, with inconsistent changes in other compartments in the most severely affected placentae. Two animals (V31 and V36) which did not abort following challenge with strain 76223 showed no histologic abnormalities in any location in the PU.

Strain 924

Two non-vaccinated animals aborting after challenge with strain 924 showed similar histopathologic lesions to strain 76223 challenged animals including minimal to moderate fibrinosuppurative placentitis with haemorrhage most prominent in the junctional zone, but vasculitis was rarely a feature. Other compartments were inconsistently and rarely affected, though the most severe pathology in animal N28 was accompanied by a minimal to mild suppurative endometritis with haemorrhage. In the non-aborting, non-vaccinated animals challenged with strain 924, two animals which had *C. fetus* subsp. *venerealis* isolated at culture (N6 and N24) had fibrinosuppurative placentitis of mild to severe grade. Notably for animal N6 the histologic lesions were more severe than in aborting animals. Another two non-aborting animals (N4 and N19) had no histological abnormalities.

Vaccinated animals challenged with strain 924 did not abort and showed no histopathologic changes in the junctional zone. Notably mild focal neutrophilic and fibrin aggregates were noted in the labyrinth of one animal V35, and this animal was culture positive.

Strain 600

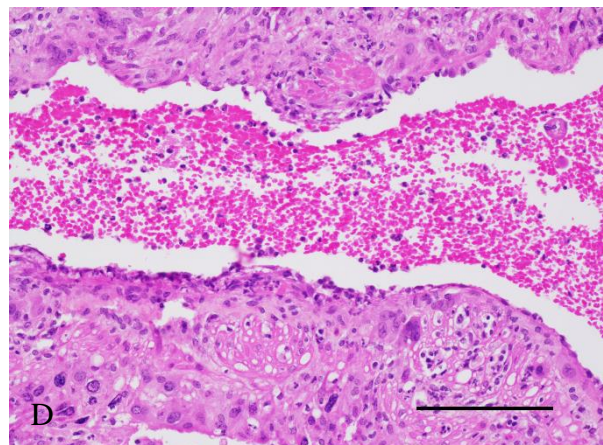
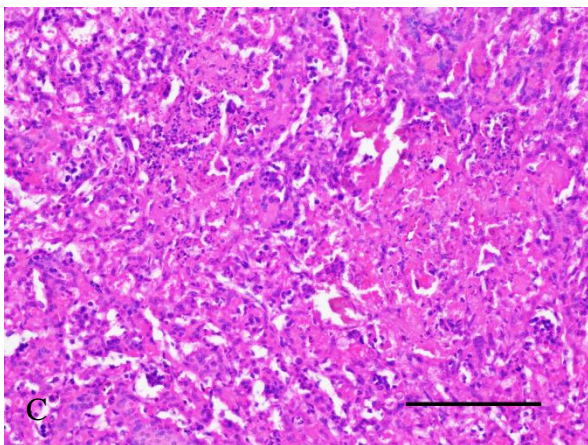
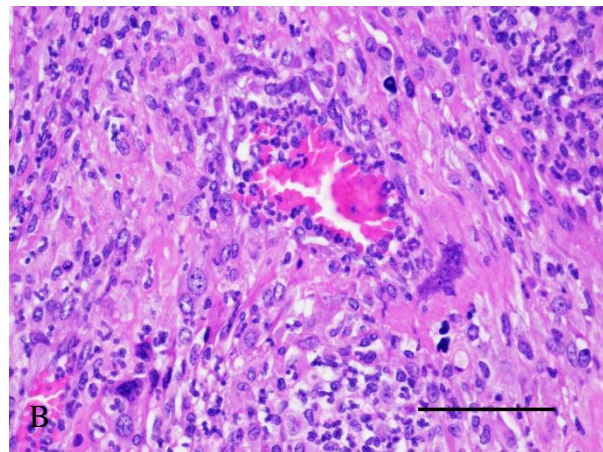
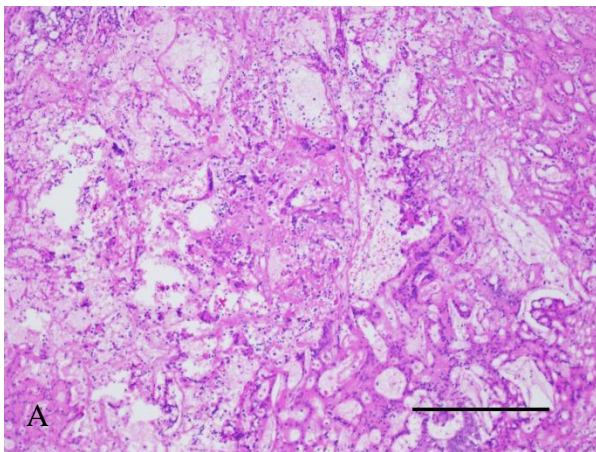
For animals challenged with strain 600, one vaccinated and one non-vaccinated animal aborted. Lesions in aborting animals were similar to those reported for other strains including minimal to moderate fibrinosuppurative placentitis with haemorrhage most prevalent in the junctional zone. Mild changes including neutrophilic margination and fibrin deposition were additionally noted in the parietal yolk sac, with some haemorrhage in the umbilical cord similar to changes noted with strain 76223. Minimal to moderate suppurative endometritis with haemorrhage were noted in the endometrium of both animals. The remaining animals, irrespective of vaccination status demonstrated no histologic changes consistent with failure to isolate any bacteria.

Strain 635

Despite causing the lowest levels of abortions, strain 635 demonstrated some of the most severe pathology of all the strains in non-vaccinated animals. Lesions were similar to those reported for

strain 76223, however were typically marked to severe in grade with fibrinosuppurative placentitis, vasculitis and haemorrhage that was most severe in the junctional zone. In this strain large areas of necrosis in the subplacenta were a prominent feature, and this was not noted in any other strain. Other placental regions were less severely and inconsistently affected but neutrophilic infiltrates, neutrophil intravascular margination, haemorrhage and necrotic debris were noted variably in the visceral and parietal yolk sac, amniotic cavity and umbilical cord, with the interlobium and labyrinth rarely affected even in severely affected placentae. Minimal to moderate suppurative endometritis was also noted in affected dams.

No vaccinated animal aborted for this strain, however moderate to severe lesions similar to non-vaccinated animals challenged with strain 635 were noted (V26 and V27), and these animals had reisolation of *C. fetus* subsp. *venerealis*. The remaining animals (V30 and V32) had no histopathologic lesions and no bacteria were isolated.



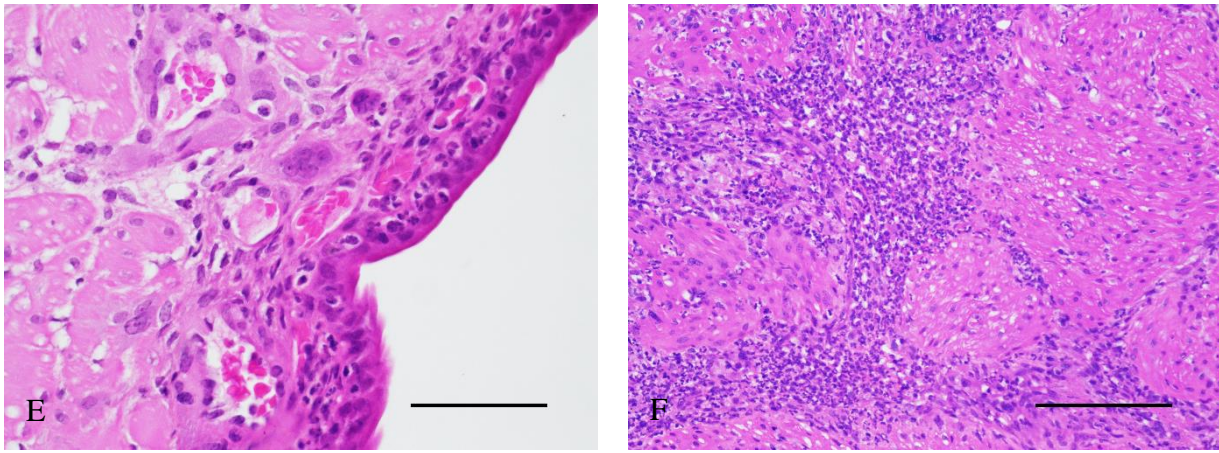


Figure 6. Histopathological changes caused by infection with *Campylobacter fetus* subsp. *venerealis* in the pregnant guinea pig model. A – An aborted discoid placenta showing necrosis and autolysis (strain 635; non-vaccinated). H&E stain; 100 μ m. B – Myometrium from a placental site of an aborting dam with grade 5 suppurative myometritis. Neutrophils are marginating within an activated blood vessel (strain 635; non-vaccinated). H&E stain; 50 μ m. C – An aborted discoid placenta with multifocal necrosis, fibrin, and neutrophilic inflammation within the labyrinth and interlobium (strain 76223; non-vaccinated). H&E stain; 100 μ m. D – Uterine artery within the myometrium of an intact placental unit with fibrinosuppurative exudate, focal fibrinoid necrosis and vasculitis (strain 76223; non-vaccinated). H&E stain; 100 μ m. E – The endometrial surface adjacent to a placental unit showing mild neutrophilic infiltration and transmigration (strain 76223; non-vaccinated). H&E stain; 50 μ m. F – A placental site from an aborting dam showing myometrial tissue with severe suppurative myometritis (strain 635; non-vaccinated). H&E stain; 100 μ m.

Inflammation scores, used as an indicator of histological response appeared to be proportional to time to abortion (Figure 7).

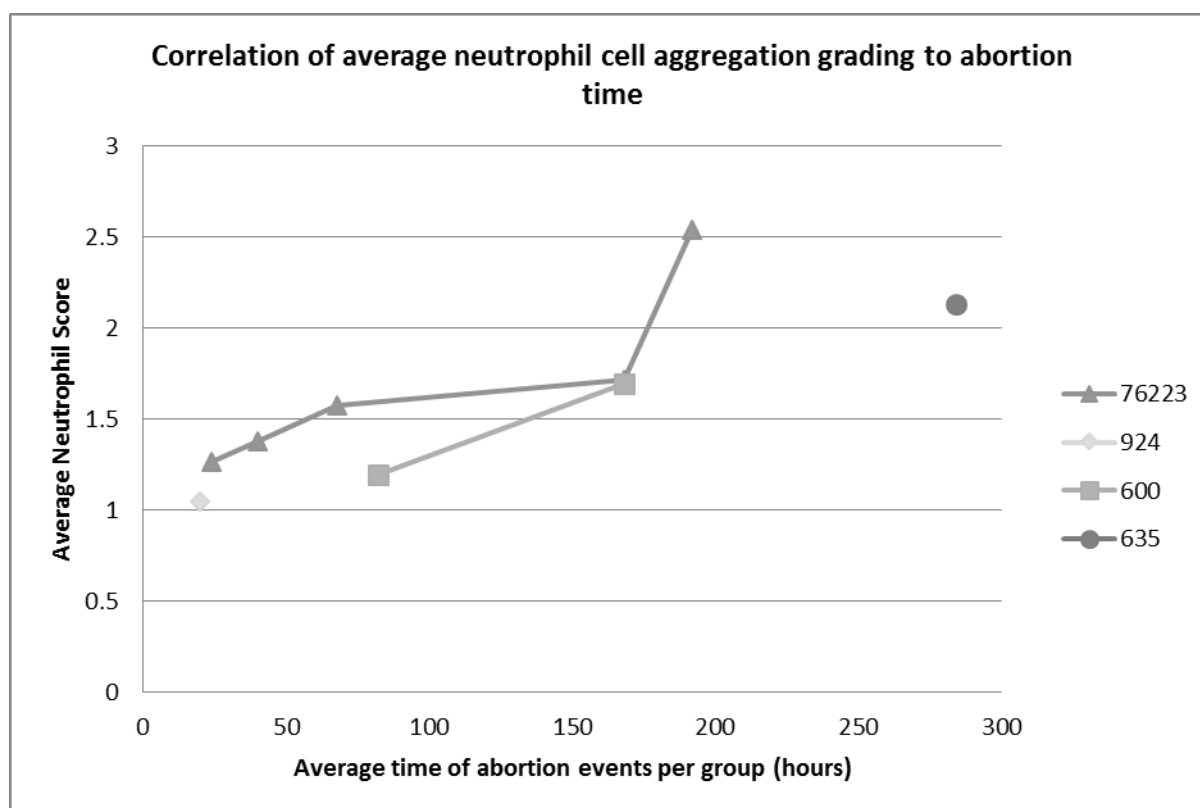


Figure 7. Average histopathological neutrophilic inflammation score per aborting dam in Experiment 2 from vaccinated and non-vaccinated dams for the 4 groups inoculated with different strains of *C. fetus* subsp. *venerealis* (strain 600, 635, 924 and 76223) over study timeline (288 h).

Discussion

This study was successful in applying a previously developed pregnant guinea pig model for the identification of *C. fetus* subsp. *venerealis* strain variation. This was determined through differences in abortion rates and infection progression as shown by bacterial culture, histopathological indicators and molecular detection. This study is novel in providing a comprehensive view of the histopathology associated with different *C. fetus* subsp. *venerealis* strains as well as identifying the most accurate and consistent methods for detection of infection.

Small animal models for the assessment of *Campylobacter* strains have been used for over 60 years (Ristic and Morse 1953, Coid, O'Sullivan et al. 1987, Burrough, Sahin et al. 2011) with few focusing specifically on *C. fetus* subsp. *venerealis* (Ristic and Morse 1953, SultanDosa, Bryner et al. 1983, Bryner, Firehammer et al. 1988) or the use of histology to identify infection (Ristic and Morse 1953, Morse and Ristic 1954, Ristic, Wipf et al. 1954). This study used a multi-faceted approach to identify infection progression for different strains in the pregnant guinea pig model. It supported the use of culture as the gold standard indicator with histopathological findings to be supplementarily indicative.

Although there was no statistical difference between the dose related abortion events in Experiment 1, there was a single abortion incidence in the 1:2.5 dose group after inoculation with a previously determined abortifacient strain (Koya, de Wet et al. 2015). The abortion occurred very early (26 h) with no indication of bacteraemia, but reculture from the uterus, placental sites and a single foetus. Overall placental tissues displayed moderate histopathological inflammation which was greater than that seen in non-aborting dams. Abortion events appeared to correlate with inflammation severity across all four dose groups, with histopathological indicators of inflammation most severe in aborting dams with only four non-aborting dams not showing any changes (2 dams, dose 1:0; 2 dams, dose 1:2.5). They were comparatively lower or equal in severity to those of the aborting dams and may be strain specific. Experimental vaccine dose dilution of 1:2.5 was therefore used in Experiment 2 as an indicator of strain virulence.

Vaccine efficacy as a marker for virulence was shown to be a reliable indicator across the four strains tested in this study and was variably protective (50% - 100%). Strain 76223, appeared to be the most virulent, with all non-vaccinated and half of the vaccinated dams aborting. The bacteria was recultured from all aborting dams, and had moderate histopathological changes associated with infection. General histopathological changes were consistent across all strains being a

fibrinosuppurative placentitis and endometritis, sometimes with features of vasculitis, thrombosis and haemorrhage indicating vascular damage. Further, there was a strong correlation between culture positive animals and histopathological changes; with no pathology seen in animals when bacteria failed to be isolated.

In Experiment 2, it was notable that the severity of histopathological changes in the PU and aborted placenta correlated with abortion time in three groups (76223, 600, 924), however, some of the most severe pathology was seen in strain 635 which had one of the lowest abortion incidents. With strain 635, the severity appeared dependent on the increased time required to induce a pathological response and develop histological lesions, showing pathology correlated with infective time, not abortion events. Previous studies have demonstrated different histological characteristics of infection, which is likely due to pathogenic differences of strains, inoculum concentrations and volumes (Ristic and Morse 1953). An experimental bovine infection study found histological changes in 5 of 6 heifers inoculated with 4.7×10^8 CFU/ml of '*V. fetus*' biovar *venerealis* isolated from an aborted foetus (Estes, Bryner et al. 1966). The study identified varying levels of mucopurulent endometritis with exudate distension consisting of primarily neutrophils, as well as lymphocytes, eosinophils and necrotic epithelial cells. Hyperaemia, hyperplasia, vascular disturbances, cellular debris and cells with hydropic degeneration and pyknotic nuclei were also visualised in uterine glands and lumen (Estes, Bryner et al. 1966). The study also found that culture correlated with histology with one uterus culture negative heifer displaying no endometrial histopathological changes (Estes, Bryner et al. 1966). While none of the heifers in this study were pregnant, and therefore offered no placental tissue for examination, many of the characteristics of uterine inflammation corresponded with that seen within the guinea pig uterus. A study into the histopathologic infection of aborted foetuses due to *C. fetus* subsp. *venerealis* infection found placentitis characterised by neutrophils and necrosis in all samples (n=2) (Campero, Anderson et al. 2005). While a small sample size, the results suggest placental inflammation as a factor of infection mechanism as seen within this study.

Differences seen in strain virulence could be attributed to inherent pathogenicity or due to genomic adaptations. Laboratory cultured strains have been shown to adapt to continuous passage by losing plasmids or phages associated with virulence. Strain 76223 was assumed to be pathogenic, due to isolation from an abortion event, however, its identification in proximity to this study allowed its inclusion after minimal subculturing. Strains 600, 635 and 924 were passaged *in vitro* numerous times prior to their use *in vivo*, which may have affected their virulence during this experiment.

Further, no correlation appeared to be apparent between virulence and the tested pathogenicity genes of these strains, though further genomic analysis is required.

Though vaccine efficacy was not an aim of this study, rather vaccination as a marker for virulence, the results are indicative of reduced efficacy in guinea pigs with only two strains producing no abortions in the vaccinated group (strain 924, 635). However, there was only reisolation of the inoculated bacteria in 2/4 non-aborting vaccinated dams for strain 635, and in none of the 9 remaining non-aborting vaccinated dams across the other three strain groups, indicating a level of protection leading to clearance of the bacteria within the timeframe (288 h) of the trial. The four aborting vaccinated animals were consistently positive across all markers for infection (i.e. culture, histopathology and PCR). Further studies specifically into vaccine efficacy would need to determine whether the rate and level of infection seen in these vaccinated animals is representative of vaccination outcomes in cattle.

No previous small animal studies have used molecular detection in the model to determine infectivity and progression. Comparative analysis of the two PCR methods appears to show a high level of specificity though questionable sensitivity in both assays with the *cstA/parA* PCR (Hum et al) identifying 10 truly positive samples with both bands (142 and 764 bp). The NahE qPCR was unable to detect bacterial DNA in 6 culture positive non-vaccinated animals, with the Hum PCR unable to detect a total of 12 culture positive animals (vaccinated and non-vaccinated). Culture and histopathology were both consistent in identifying infected dams in this study; however, molecular testing may require further validation. A number of culture positive non-vaccinated animals (n=5) were not detected by either PCRs, which could be due to variations in sampling sites for each method, or due to the non-quantification of DNA prior to testing affecting detection. *CstA/parA* PCR results where both bands were present corresponded with culture, histology and/or NahE qPCR positives. However, a large number of samples produced only one band in the *CstA/parA* PCR (27.4% of 'suspect' samples produced only the 142 bp band; 57.1% only the 764 bp band), requiring the sample to be either retested or concluded as negative. Only one sample which produced the two bands required, had no other indicators of infection by other testing methods (i.e. culture, histology or NahE PCR). This discrepant result was from a dam spleen sample from strain 600, and could be due to DNA from dead bacteria clearing through the immune system.

This study was limited in the number of strains selected for use to minimise the research impact on animals. However, further studies into *C. fetus* subsp. *venerealis* could determine virulence markers to be tested *in vitro* to enable a better understanding of strain pathogenicity. Future research could

include the examination of virulence gene mutant strains and their effect on pathogenicity as previously examined using a cell culture virulence model (Kienesberger, Gorkiewicz et al. 2007). This could be useful in diagnostic laboratories for the determination of bacterial pathogenesis in mixed infections, as well as in vaccine development. Further, the study has expanded on our knowledge of the histopathological changes induced by *C. fetus* subsp. *venerealis* in a guinea pig model, including correlation with previously reported changes in cattle, and the relationship of histopathology to abortion kinetics. This small animal model for the study of *C. fetus* subsp. *venerealis* infection and strain variation has successfully indicated the presence of variation in Australian strains of *C. fetus* subsp. *venerealis* using vaccination as a virulence marker.

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Chapter 8. General Discussion

8.1 Introduction

BGC is a disease of significant economic impact in large cattle farming countries. Traditionally, diagnosis of disease is through culture of the causative organism, *C. fetus* subsp. *venerealis*. Culture has some limitations, as there are difficulties in isolation and identification of the bacteria. Despite these limitations, culture remains the ‘gold standard’ for declaration of freedom of disease for export purposes (OIE 2012). It is the only method of distinguishing between the two biovars, and currently, a more reliable and cheaper method of distinguishing between subspecies than molecular testing.

Biochemical differentiation of subspecies and biovars are reliant on single tests; identification of *C. fetus* subspecies relies on glycine tolerance while biovar identification relies on production of hydrogen sulphide in a cysteine medium. These biochemical tests are unreliable as glycine tolerance can be acquired or lost through phage transduction (Chang and Ogg 1971). Hydrogen sulphide production causes a colour change on lead acetate paper in the presence on cysteine metabolism which is subjective in interpretation (Veron and Chatelain 1973). Current comparative research into phenotypic characterisation with molecular identification has shown that culture diagnosis is still inadequate compared to genomic identification and improvements are clearly required to continue its use as the gold standard (van der Graaf-van Bloois, Miller et al. 2014).

Some strains produce anomalous results on other differentiating characteristics, including thermo-tolerance and antibiotic susceptibility. All *C. fetus* strains are considered to be negative for growth at 42°C according to OIE guidelines, with variable tolerance of 25°C (OIE 2012). However, studies have identified thermophilic *C. fetus* strains exist in both human and cattle samples (Klein, Vergeront et al. 1986, Schulze, Bagon et al. 2006). It is not known whether variation in these biochemical traits is associated with virulence. At the molecular level pathogenicity islands carrying genes for resistance to tetracycline and streptomycin have been identified in *C. fetus* subsp. *fetus* strains (Abril, Brodard et al. 2010). Identification of such traits may allow differentiation of strains associated with clinical disease status compared with infectious status.

The OIE specifications for isolation and identification of *C. fetus* subsp. *venerealis* strains were adapted from the 2nd edition of Bergey’s Manual published in 2005 (Vandamme 2005). Advancements in molecular and microbial methods based on the literature in the last 10 years needs to be included and current best practice evaluated and updated.

Experimental infection small animal models allows for assessment of clinical disease compared to *in vivo* studies. Small animals (guinea pigs and hamsters) have previously been used with success for the infection of *Campylobacter* species (Ristic and Morse 1953, Ristic, Morse et al. 1954, Ristic, Wipf et al. 1954, Bryner, Foley et al. 1978, Bryner, Foley et al. 1979, SultanDosa, Bryner et al. 1983, Taylor and Bryner 1984, Coid, O'Sullivan et al. 1987, Bryner, Firehammer et al. 1988, Burrough, Sahin et al. 2009, Burrough 2011, Burrough, Sahin et al. 2011). While there are differences in study design, establishment of infection and production of similar clinical signs suggest that small animal models may be useful for the assessment of strain virulence. As there are no current biochemical or molecular markers that are able to determine the virulence of strains, the use of *in vivo* studies can allow the identification of virulent strains for further study.

There is therefore a need to evaluate current enrichment and isolation methodologies available for *C. fetus* subsp. *venerealis* and identify the level of variation in biochemical characteristics; with the ultimate aims of i) identifying new more reliable assays for differentiation of subspecies or biovars and ii) assessing the reliability and reproducibility of biochemical testing methodologies and iii) developing a reproducible, reliable small animal model with defined characteristics to characterise strain virulence variation *in vivo*.

Objective 1. Identification of optimal transport and isolation methodologies for *C. fetus* subsp. *venerealis*

The aim of this objective was to compare the newer developed TTE with the commonly used Lander's TEM for the enrichment of *C. fetus* subsp. *venerealis* isolates in an *in vitro* spiked mixed culture laboratory trial and an *in vivo* cattle trial. It also aimed to identify best practice for isolation via either *Campylobacter* selective agar or using non-selective SBA with or without a filter. The influence of incubation temperature on *C. fetus* subsp. *venerealis* was also studied, with differential incubation at 25°C compared to 37°C. Both media appeared to produce comparable isolations from both trials, while subculture onto *Campylobacter* agar produced the most consistent isolations in the presence of mixed cultures. Direct plating onto SBA allowed overgrowth of contamination while the use of a filter required a high starting concentration and results in variable isolations.

The emphasis on culture as the gold standard for diagnosis is indicative of the importance of optimal methodologies being implemented. While molecular testing has been shown to be more sensitive, its specificity is questionable and there are still no current reliable targets for *C. fetus*

subsp. *venerealis* strains. Molecular testing should also be carried out on pure culture extractions as boiled lysates have been shown to produce false positives. Immunological testing has a reduced sensitivity and specificity and is currently only recommended as herd testing. This further emphasises the need for accurate culture methods. Current BGC national (ANZSDP, Hum, 2009) and international (OIE, 2012) protocols specify guidelines for the collection of samples, transport, isolation and identification of *C. fetus* subsp. *venerealis* bacteria. Some specifications are general to allow adaptation by different laboratories, to media or sampling tools they might have available.

The guidelines suggest the use of a transport and enrichment medium (TEM) for samples that cannot be delivered to diagnostic laboratories within six h (Hum, 2009), or on the same day of collection (OIE 2012). In Australia, samples are often in transit for up to 72 h due to the distance of farms from diagnostic laboratories.

TEMs currently in use and included in the OIE guidelines (OIE 2012) include Lander's, Clark's or Cary-Blair's media. The newer Thomann Transport and Enrichment (TTE) medium (Harwood, Thomann et al. 2009) developed in 2009 has not been included in any OIE protocols, because to date there are no published comparisons of its performance in relation to the currently specified media. The potential for discrepant results based on controlled conditions of a laboratory trial compared with that of a field trial were accommodated for in this study, and therefore, both media were tested in both environments.

The primary aim of this study was to compare the performance (number of isolations) of TTE and the frequently used Lander's using a spiked mixed culture experiment with the secondary aim of comparing selective agar to filtration for optimal culture success. A second study compared the two TEMs (TTE and Lander's) in a large animal infection trial to determine differences in isolation success in a controlled field trial.

In the laboratory experiment (Chapter 4) TTE and Lander's resulted in isolations from the same samples, and from pure cultures incubated up to 168 h. In cultures spiked with *P. vulgaris*, *C. fetus* subsp. *venerealis* was isolated after 48 h from samples incubated in TTE on Campylobacter agar, contrasting with 24 h for samples from Lander's medium using filtration. The latter media and culture process could facilitate shorter culture reporting times or, in the event of contamination from a combination of other bacteria (species) allow isolation prior to becoming completely overgrown.

TTE also allowed the isolation of *C. fetus* subsp. *venerealis* from *C. jejuni* spiked cultures incubated at 37°C on SBA and Campylobacter selective agar, while Lander's only allowed isolation on SBA. This is of particular importance locally where it has become evident that contention from thermophilic *Campylobacter* isolates can be problematic. This issue has not been raised in the literature previously and may be a geographical issue due to temperature and humidity caused by the subtropical climate (Chapter 4). With transport of samples over long periods and the potential for increased contamination resulting from this, the use of selective agar may be more favourable. With the inability of samples to grow on Campylobacter selective agar from Lander's, this could affect culture success if both agar plates are not utilised in the culture process.

Overgrowth of *C. fetus* subsp. *venerealis* cultures by thermophilic *Campylobacter* species could be overcome by modification of the current protocols for incubation of samples at 37°C to 25°C. *C. fetus* subsp. *venerealis* is able to grow at 25°C, while most thermophilic *Campylobacter* species are not. We found that incubation of samples at 25°C and to 37°C did not negatively impact on the enrichment of *C. fetus* subsp. *venerealis*, but improved enrichment and isolation in the presence of spiked bacteria; *P. vulgaris* and *C. jejuni*.

Previous studies have favoured Lander's TEM over Clark's or Carey-Blair's media due to higher isolation rates (Lander 1990, Monke, Love et al. 2002). The simplicity of the Lander's medium production compared to Clark's also makes it a much more favourable option (Hum, Brunner et al. 1994). Comparison of ease of production and implementation for Lander's and TTE favours TTE due to the lower number of reagents and the lack of charcoal and horse blood, which also allows it to be used for direct molecular testing. While molecular testing was not included in this study, for diagnostic laboratories where concurrent molecular and bacteriological testing is performed on samples submitted, using a single sample would reduce media costs as well as any potential discrepancies seen when testing two different samples by two different methods. Further TTE has reduced antibiotic concentrations for vancomycin, trimethoprim and polymyxin B compared to Lander's which may decrease the selection for resistant strains only.

The use of selective agars or filter exclusions are frequently used to improve isolation of *Campylobacter* species from different type of samples (preputial fluid, vaginal mucus, faecal matter) (Kulkarni, Lever et al. 2002, Speegle, Miller et al. 2009, Bessède, Delcamp et al. 2011, Chaban, Guerra et al. 2013). Previous studies have shown that for isolation of *C. fetus* subsp. *venerealis* Campylobacter selective agar performed better than Karmali or Skirrow's agar (Hum,

Brunner et al. 1994, Monke, Love et al. 2002, Indjein 2013). Comparison of Campylobacter selective agar (Oxoid) with filtration (0.45 µm) in this study found the former produced more consistent positive results regardless of incubation temperature. Filtration required a much higher starting concentration of *C. fetus* subsp. *venerealis* (minimum 10⁵ CFU/ml) and therefore risks producing false negative culture results from samples with low concentrations. Campylobacter agar was able to produce growth from *C. jejuni* and *P. vulgaris* spiked cultures, while also allowing distinction between the two species on the agar, while SBA plates allowed overgrowth in *P. vulgaris* spiked cultures. This is particularly useful given the likelihood of sample contamination and for diagnostic laboratories or research where multiple species are screened for.

There was no significant difference in the number of *C. fetus* subsp. *venerealis* strains isolated from the cattle in this study from either TTE or Lander's. There were significantly more non-*C. fetus* subsp. *venerealis* *Campylobacter*-like strains isolated from TTE than from Lander's medium. This could be due to the lower concentration of antimicrobials in TTE, allowing more bacterial growth. There were limitations with the study involving the unknown infection status of the cattle, however, based on the isolation rates of *C. fetus* subsp. *venerealis* overall, either tested media was able to adequately support growth and enrichment of strains during sampling and transport within this field trial.

Based on the results of these studies, optimal transport conditions could be achieved using either media, TTE or Lander's TEM for submission of diagnostic samples. Further, TTE would allow for both microbial and molecular testing of the same sample if necessary. These studies showed that while filtration allowed isolation from spiked mixed cultures, the required high starting concentration is not ideal for samples from animals with low infective status, leading to possible false negative results. SBA plates are useful only for minimally contaminated samples, while *P. aeruginosa* and *P. vulgaris* spiked mixed cultures produced no *C. fetus* subsp. *venerealis* growth in this study. Overall, Campylobacter selective agar allowed optimal isolations by producing the most common, consistent positive results in the presence of each simulated contaminant. Therefore, the preferred option for culture media is Campylobacter selective agar, however, multiple culture options may increase the chances of producing positive *C. fetus* subsp. *venerealis* isolations.

Objective 2. Phenotypic characterisation: A comparison of culture phenotyping and automated methodologies

The aim of this objective was to phenotypically characterise 152 *Campylobacter* isolations according to OIE recommended methods for *C. fetus* subsp. *venerealis* in order to determine the level of reproducibility and variation in tests and results. It also aimed to compare the identification of cultures phenotypically with automated tests including API Campy kits, Biolog GEN III microplates and Biolog Phenotypic MicroArrays. Expansive antibiotic profiling of *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis* and *C. hyointestinalis* identified multiple antibiotics that may be implemented as differentiating tests. It was found that there were failings in the implementation of certain phenotypic tests, including oxidase and glycine tolerance with a high number of isolates molecular profiled as *C. fetus* subsp. *venerealis* returning oxidase negative results. Two known *C. fetus* subsp. *fetus* strains were negative for growth in 1% glycine. Use of API kits produced a low level of agreement between phenotypic characterisation of *C. fetus* subsp. *venerealis* strains, while *Arcobacter* strains were correctly identified. Biolog GEN III identified methyl pyruvate substrate for the potential difference of *C. fetus* subsp. *venerealis* biovars while the PM plates had poor reproducibility.

Biochemical characterisation of isolated strains can be inconsistent depending on inoculum size or testing methodologies. *C. fetus* subsp. *venerealis* is characterised by microaerobic growth, corkscrew motility and a number of recommended OIE differentiating tests (OIE 2012). The reproducibility of the majority of these tests or the effects of inoculum size has not been previously studied. A study was conducted into the reproducibility of a variety of biochemical tests on *Campylobacter* species including one *C. fetus* subsp. *venerealis* strain (On and Holmes 1991). These included 1% glycine and antibiotic and temperature tolerances, where it was found that in general there was some level of non-reproducibility across most tests. This was dependent on the basal medium used for testing, regardless of consistency in culture age or inoculum size (On and Holmes 1991). The effect of the inoculum size on test results has also been investigated, with the only one relevant to *C. fetus* subsp. *venerealis* being 1% glycine tolerance (On and Holmes 1991). The study found that inoculum size of 10^6 CFU/ml was necessary to produce reliable results, while 10^8 CFU/ml produced false positive results with the strain tested (On and Holmes 1991).

In this study consistency was aimed for with each biochemical test. All tests requiring agar were carried out using SBA plates (Oxoid) and tests requiring fluid media were conducted in

Campylobacter broth (Chapter 3). Standard inoculum sizes were used for each test with all testing conducted on cultures of the same age. Strains were consistently grown for 72 h to produce a concentration of approximately 10^7 CFU/ml which was then used to inoculate test media. The variations in growth rates, especially for strains resuscitated from storage, which were noted to be slower growing, could have affected the test results. False positive glycine results were not an issue noted in our research, with more frequently, glycine negative results identifying strains as *C. fetus* subsp. *venerealis* when MLST typing identified them as *C. fetus* subsp. *fetus* (Chapter 5).

Standardized inoculation concentrations and volumes are potentially of greater importance than has previously been noted, with OIE guidelines noting a cell suspension of approximately McFarland no. 1 should be used for most assays, with no mention of inoculation volume. As colony counts which are time consuming are not a viable option prior to biochemical characterisations, a turbidity check using a McFarland standard as minimal to ensure there is a sufficient concentration of organisms may be necessary. Further, strains could be submitted to concurrent molecular testing from genomic DNA to ensure accurate identification. The diagnostic isolation rate for *C. fetus* subsp. *venerealis* appears to be low, therefore strain confirmation would not be an excessive burden on laboratory processes.

While *C. fetus* strains appear to have a similar morphology, thermophilic *Campylobacters* appeared to have a distinct 'green' tinge on SBA and Campylobacter agar plates. The colonies also appeared to become slightly larger comparative to *C. fetus* subsp. *venerealis* over the 72 h incubation period at 37°C. While this is purely an observational finding based on the strains isolated and resuscitated in this study, it may warrant further research to allow easier distinction of varying strains earlier in the identification process.

The high presence of thermophilic *Campylobacters* found in the field trial as well as during biochemical characterisation of the strain collection is not only indicative of the potential geographical differences in *Campylobacter* presence, but also the need for identification and characterization of bacteria found in the penile sheath and the vaginal flora of cattle. It also emphasizes the importance of considering all *Campylobacter* species when conducting differentiating tests. *Arcobacter* species are commonly able to tolerate microaerobic atmospheres as well as growing aerobically at temperatures below 40°C (Schroeder-Tucker, Wesley et al. 1996). If aerobic control plates are not run in parallel during testing, the species can easily be confused with *C. fetus* subsp. *venerealis* as has occurred previously (Indjein 2013). The minimal information

regarding *Arcobacter* (and other *Campylobacter* species) in veterinary diagnostic laboratory protocols means they can be easily overlooked as a contaminant during diagnosis. Not only do laboratory workers need to be aware to include them in differential testing consideration, but veterinary pathologists need to be mindful of their potential involvement in disease and question their presence in differential diagnosis.

API Campy kits were found to be unsuccessful in identifying *C. fetus* subsp. *venerealis* strains and while did correctly identify three tested *Arcobacter* strains, the kits also incorrectly identified a number of other strains comprising different species as *Arcobacter*. Therefore, using the kit for the identification of *Arcobacter* species only may lead to false positives. Phenotypic characterisation was found to be the most reliable identification method for *C. fetus* subsp. *venerealis* strains and while newer testing methodologies including Biolog GEN III and PMs include an array of differentiating tests, the lack of a conclusive *C. fetus* profile means that while they could be used as research tools to identify substrates which allow/ do not allow respiration, they are unable to be implemented diagnostically.

While the testing of Biolog technologies aimed to identify tests that may have been useful in distinguishing species and subspecies, the limited number of strains tested and lack of reproducibility across certain tests did identify any unique identifiers using the Biolog PM system. The GEN III system identified methyl pyruvate which appeared to agree with H₂S testing independently (Chapter 5). Due to the subjectivity of the H₂S test and the inability to identify *C. fetus* subsp. *venerealis* biovars by any other biochemical or molecular methods, it is unknown how accurate this identification is. Similarly, test results that did not align between independent H₂S and GEN III methyl pyruvate could have been due to failings of the biochemical testing methodology, of which there is no confirmatory testing. Further research is needed to determine if a methyl pyruvate substrate could be implemented in a complimentary identification test for diagnostic laboratory use to aid in biovar identification. The differentiation of biovars is not of any apparent significance as diagnosis with either biovar is considered indicative of BGC and carries the same implications. Distinction between the two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* is of greater importance as the former is not causative of BGC and therefore is less restrictive in its diagnosis. However, the differentiating test can be equally subjective and depending on inoculum size (On and Holmes 1991) and genomic alterations (Chang and Ogg 1971) can lead to false results. Due to the limited number of *C. fetus* subsp. *fetus* strains available in this study, the ability to identify differentiating tests was restricted. There did however appear to be

differences in antimicrobial susceptibility between the two subspecies, though the results were limited by the low number of strains tested (Chapter 5). If further studies could expand these trends to a larger number of strains they may be useful in adding a secondary differentiating feature to subspecies testing.

It was evident from the results of phenotypic characterisation that more research is required into the ideal inoculum size and concentration for *C. fetus* subsp. *venerealis* differentiating tests. The potential false negative identifications seen with strains where oxidase returned a negative result indicating that a higher inoculum size is needed for testing. Similarly, false negative glycine test results for two of the *C. fetus* subsp. *fetus* strains questions the method as well as any potential genomic alterations that may have occurred either during storage or passaging of the strain since its attainment from the UK laboratory. National and international protocols do not provide explicit details of testing methodologies and are open to variations which can lead to differences in subsequent results. Due to the unique growth specificities and sensitivities of *C. fetus* strains, these details may have a large impact on the accuracy of identification of isolated strains.

The Biolog PMs contained a range of antimicrobial sources of which there were four different concentrations tested. The results were inadequate to define trends between species or subspecies due to limited number of strains tested, however, if the testing methodology could be adequately refined, it may provide a research tool for identifying variations in antibiotic concentration tolerances between species, subspecies and biovars. The Biolog PMs could also be used to identify virulence characteristics by testing knockout strains and determining the change in assay metabolisms. Currently, minimal *in vivo* and *in vitro* studies are evident in the literature to allow better understanding of strain variations. Molecular testing for identification of pathogenicity islands or virulence factors that may relate to pathogenicity have identified antimicrobial susceptibility islands in *C. fetus* subsp. *fetus* strains for tetracycline and streptomycin resistance (Abril, Brodard et al. 2010). Testing of all strains for susceptibility to either antibiotic found only two *C. fetus* subsp. *venerealis* strains that showed resistance to either antibiotic. Testing of one strain (924) with resistance to both antibiotics in a small animal model did not identify any higher level of pathogenicity comparative to the other three strains tested.

While these studies did not identify any new identification tests or methods, the development of a comprehensive profile of Australian *Campylobacter* isolations from preputial or vaginal mucus samples has allowed the identification of tests which are most likely to fail or produce discrepant

results. In this study, oxidase testing produced the highest number of variable results, with a large number of strains testing negative. Reagents were quality checked once a week, therefore, the failing is based on implementation of the test, in this case a smaller than required inoculum size. It is indicative of the importance of consistent parameters for each test, with further research is required to determine the ideal inoculum sizes necessary for each test, and the likelihood of producing false results. Antimicrobial susceptibility testing showed promising potential for identifying differentiating tests between *C. fetus* subspecies and with further research, may also allow differentiation between other commonly isolated *Campylobacter* strains from cattle. API kits and Biolog technologies at this time do not afford any improvements in identification of *C. fetus* subsp. *venerealis* strains. API kits failed to identify strains, although *C. fetus* is currently included in the identification database, while Biolog has not yet produced a profile for *C. fetus* in its database. The identification of individual differentiating tests in the Biolog plates was not successful with the PMs due to poor reproducibility, potentially due to strain alterations during storage. The GEN III technology did identify methyl pyruvate as a potential source for biovar distinction within the same metabolic cycle as cysteine producing H₂S. These studies have shown the importance of considering the presence of other *Campylobacter* species in cattle samples with a large number of non-*C. fetus* subsp. *venerealis* strains identified during this research. Phenotypic characterisation still remains the most reliable method for identification of *C. fetus* subsp. *venerealis* strains.

Objective 3. Development of small animal model for the assessment of Australian *C. fetus* subsp. *venerealis* strain variation

This objective aimed to produce a reliable pregnant guinea pig model for the assessment of *C. fetus* subsp. *venerealis* strain virulence variation. An initial model was developed with defined parameters for animal inoculation as well as outcome monitoring. This was used to determine the ideal dose of an experimental vaccine for use as a parameter for virulence identification in four characterised *C. fetus* subsp. *venerealis* strains. It was found that a concentration of 10^7 CFU/ml inoculated via the IP route to pregnant guinea pigs at week 5-6 of gestation could produce comparable outcomes of abortion time, abortion rate, re-culture of the inoculation bacterium, identification of infection of infection progression through molecular and histopathological analysis of uterine and placental tissue.

Identification of strain variations in a guinea pig model required the evaluation of the parameters involved to identify a reliable and reproducible model (Chapter 6). An initial trial was conducted to determine the correct dose, route and timeline for the study. Testing of three different doses (10^4 , 10^7 and 10^9 CFU/ml) found that the lowest dose was unable to produce abortions or any indicators of infection, indicative of it being below the minimal infective dose. A concentration of 10^7 CFU/ml was found to be a reproducible concentration producing 75% abortions using the same strain (540) at two different time points (Chapter 6 and Chapter 7 Experiment 1). The average time to abortion was comparable as well with IQR of 68 h in the trial (Chapter 6) compared to 65 h in the dose study (Chapter 7).

The trial was already indicative of strain virulence variation presence with three strains (258, Q41 and 540) tested showing different abortion rates. While strain 258 was able to produce 100% abortions at a dose of 10^9 CFU/ml, Q41 was unable to produce any abortions at 10^7 CFU/ml within 168 h. Whether this was due to an inadequate time allowed for abortion or due to the loss of any potential virulence genes due to age (BSL *C. fetus* subsp. *venerealis* reference strain) is unknown. However, strain 540 was able to produce 75% abortions via IP inoculation and 0% via IVA inoculation. This was indicative of failings of the inoculation method with no passage of the bacteria through the cervix, rather than the strain, although two previous studies have successfully produced infection/abortion in guinea pig models via IVA inoculation (Ristic and Morse 1953, Ristic, Morse et al. 1954). Both of these studies utilised a much higher volume of 1 ml administered on five alternate days. This resulted in 17 – 20% abortions. In the first study the same strain was

able to produce 57% abortions via IP inoculation, though over a longer time period of 25 d compared to 18 d for IVA inoculation (Ristic and Morse 1953).

Initial histological examination of *Vibrio fetus* infected guinea pigs found that foetal and dam tissues displayed extensive necrosis, oedema, haemorrhage and placentitis (Ristic and Morse 1953). Our study found that placentitis and endometritis were common features in aborting dams with severity dependent on strain and time to abortion. The much more severe pathological findings in the initial studies could also be due to the higher volumes or doses administered. The initial findings of neutrophil aggregation at placental sites and inflammation of the uterus seen in the trial (Chapter 6) were expanded on in the strain variation study (Chapter 7) with histopathological markers of infection extensively described. Findings of vasculitis were noted as markers of strain pathogenicity with strain 635 producing the most severe histological inflammation due to the longest time to abortion. However, based on overall abortion rates it was not the most pathogenic strain. Strain 76223 which was isolated from an abortion event in SE QLD was found to be the most virulent with 100% abortions in the non-vaccinated group. While its virulence was assumed, due to the nature of its previous disease pathogenesis, it was also stored for a short amount of time and with minimal subculturing prior to inclusion in the study. This potential impact of time stored or number of sub-cultures before genomic changes occur needs to be further investigated through genomic studies, or if Biolog PMs could be proven reliable and reproducible they might offer some indicators of alterations in bacteria over continuous subculturing.

The Biolog PM duplicated results have already indicated that strains may potentially change after storage and resuscitation over certain characteristics with carbohydrate utilization appearing fairly conserved over the duplicate testing at two different time points while chemical assay substrates produced a large level of variation when duplicated. However, strain 540 which was used in the initial guinea pig trial (Chapter 6) followed by storage at -80C to be resuscitated approximately 1 year later for use in the strain variation study showed no alteration of biochemical characteristics or on abortion rates.

Identification of strain variation in the guinea pig model using vaccine efficacy as a marker for virulence has identified strains of varying pathogenicity in non-vaccinated (17 - 100% abortive) and vaccinated animals (0 - 50% abortive). The pathology data of these strains could be useful when interpreting genomic analysis. This could also further assist in vaccine development by including strains identified as possessing a wide range of these virulence makers. While it is important to

stress that this was not a vaccine efficacy study, but rather the use of the vaccine as an indicator of virulence, it could be speculated that the reduced protection of the trialled experimental vaccine in the guinea pig model could also have a reduced efficacy in cattle.

The concordance of these results with a large animal study would confirm that the small animal studies, are acceptable, not only for assessing vaccine efficacy but for gaining a better understanding of the association of infection factors and outcomes in guinea pigs compared to cattle. The pregnant guinea pig model is a promising model for the study of *C. fetus* subsp. *venerealis* infection mechanisms, abortion and strain virulence differences. Further studies to elucidate abortion mechanism and vaccine efficacy could increase the understanding of the infection and abortion process.

8.2 Conclusions and future directions

This thesis was able to independently assess the newer TEM, TTE compared to Lander's and found it equally adequate. This may assist laboratories where the cost or possibility of acquiring horse blood is not always feasible and allow standardisation of media used. It could also help reduce media usage and costs for laboratories conducting concurrent molecular and microbial testing, as the same tube of media could be used for both assays. It was also found that the use of *Campylobacter* selective agar allowed greater culture success than direct plating onto SBA or with a 0.45 µm filter in the presence of *P. vulgaris* or *C. jejuni* spiked mixed cultures. Further, due to the prevalence of thermophilic *Campylobacter* species, differential incubation at 25°C compared to 37°C may assist with reducing overgrowth of not only thermophilic *Campylobacter* species, but *P. vulgaris* as well. Further research on the impact of a reduced incubation temperature on field samples is necessary to determine if it is a viable change in the presence of multiple contaminating organisms.

Identification of *Campylobacter*-like organisms predominantly from preputial or vaginal samples found that culture phenotyping was most reliable compared to API Campy kit or Biolog GEN III or PM testing. There are variabilities with the independent testing methodologies that need to be further studied and conclusively defined such as specific inoculum sizes for each test. If automated technologies such as API kits or Biolog assays are to be used in any successfully, a robust profile for *C. fetus* strains needs to be developed as well as commonly found *Campylobacter* strains, for exclusion during the differentiation process. This study has shown that the bacteria present in samples submitted for diagnosis can contain a variety of *Campylobacter* species, and in order to

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fully understand the implications of the different species further research is needed. It has also shown that more awareness of the different *Campylobacter* and *Arcobacter* species needs to be present in laboratories to allow better diagnosis and reduce false *C. fetus* subsp. *venerealis* positives. Further work on antimicrobial susceptibilities of strains would be of interest in media modifications, and potentially for differentiating tests between subspecies to support glycine tolerance testing which has been shown to be unreliable. This may also allow further research into antimicrobial resistance related to strain virulence to be explored.

A pregnant guinea pig model was successfully developed which identified dose, timeline, route and outcomes to be monitored to allow identification of strain variations. Re-culture of the causative bacterium and histopathology were found to be good indicators of bacterial progression and strain virulence, with severity of inflammation correlating with time to abortion. Vaccine efficacy as a marker for strain variation was successfully used to identify strain virulence. Strain variation as studied in the guinea pig model is evident in strains isolated from cattle in Australia. Further research into the correlation of the guinea pig model parameters and strains tested, in a cattle model may assist in the understanding of the reliability of guinea pigs as test models.

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Appendices

Appendix 1.

Biolog plate substrates as taken from MicroPlate™ handbook.

PM1 MicroPlate™ Carbon Sources

A1 Negative Control

A2 L-Arabinose

A3 N-Acetyl-DGlucosamine

A4 D-Saccharic Acid

A5 Succinic Acid

A6 D-Galactose

A7 L-Aspartic Acid

A8 L-Proline

A9 D-Alanine

A10 D-Trehalose

A11 D-Mannose

A12 Dulcitol

B1 D-Serine

B2 D-Sorbitol

B3 Glycerol

B4 L-Fucose

B5 D-Glucuronic Acid

B6 D-Gluconic Acid

B7 D,L- α -Glycerol- Phosphate

B8 D-Xylose

B9 L-Lactic Acid

B10 Formic Acid

B11 D-Mannitol

B12 L-Glutamic Acid

C1 D-Glucose-6Phosphate

C2 D-Galactonic Acid- γ -Lactone

C3 D,L-Malic Acid
C4 D-Ribose
C5 Tween 20
C6 L-Rhamnose
C7 D-Fructose
C8 Acetic Acid
C9 α -D-Glucose
C10 Maltose
C11 D-Melibiose
C12 Thymidine
D-1 L-Asparagine
D2 D-Aspartic Acid
D3 D-Glucosaminic Acid
D4 1,2-Propanediol
D5 Tween 40
D6 α -Keto-Glutaric Acid
D7 α -Keto-Butyric Acid
D8 α -Methyl-DGalactoside
D9 α -D-Lactose
D10 Lactulose
D11 Sucrose
D12 Uridine
E1 L-Glutamine
E2 M-Tartaric Acid
E3 D-Glucose-1Phosphate
E4 D-Fructose-6Phosphate
E5 Tween 80
E6 α -Hydroxy Glutaric Acid- γ Lactone
E7 α -Hydroxy Butyric Acid
E8 β -Methyl-DGlucoside
E9 Adonitol
E10 Maltotriose
E11 2-Deoxy Adenosine
E12 Adenosine

F1 Glycyl-LAspartic Acid
F2 Citric Acid
F3 M-Inositol
F4 D-Threonine
F5 Fumaric Acid
F6 Bromo Succinic Acid
F7 Propionic Acid
F8 Mucic Acid
F9 Glycolic Acid
F10 Glyoxylic Acid
F11 D-Cellobiose
F12 Inosine
G1 Glycyl-LGlutamic Acid
G2 Tricarballic Acid
G3 L-Serine
G4 L-Threonine
G5 L-Alanine
G6 L-AlanylGlycine
G7 Acetoacetic Acid
G8 N-Acetyl- β -DMannosamine
G9 Mono Methyl Succinate
G10 Methyl Pyruvate
G11 D-Malic Acid
G12 L-Malic Acid
H1 Glycyl-LProline
H2 p-Hydroxy Phenyl Acetic Acid
H3 m-Hydroxy Phenyl Acetic Acid
H4 Tyramine
H5 D-Psicose
H6 L-Lyxose
H7 Glucuronamide
H8 Pyruvic Acid
H9 L-Galactonic Acid- γ -Lactone
H10 D-Galacturonic Acid

H11 Phenylethyl- amine

H12 2-Aminoethanol

PM2A MicroPlate™ Carbon Sources

A1 Negative Control

A2 Chondroitin Sulfate C

A3 α -Cyclodextrin

A4 β -Cyclodextrin

A5 γ -Cyclodextrin

A6 Dextrin

A7 Gelatin

A8 Glycogen

A9 Inulin

A10 Laminarin

A11 Mannan

A12 Pectin

B1 N-Acetyl-DGalactosamine

B2 N-AcetylNeuraminic Acid

B3 β -D-Allose

B4 Amygdalin

B5 D-Arabinose

B6 D-Arabitol

B7 L-Arabitol

B8 Arbutin

B9 2-Deoxy-DRibose

B10 I-Erythritol

B11 D-Fucose

B12 3-0- β -DGalactopyranosyl-DArabinose

C1 Gentiobiose

C2 L-Glucose

C3 Lactitol

C4 D-Melezitose

C5 Maltitol

C6 α -Methyl-DGlucoside

C7 β -Methyl-DGalactoside

C8 3-Methyl Glucose
C9 β -Methyl-DGlucuronic Acid
C10 α -Methyl-DMannoside
C11 β -Methyl-DXyloside
C12 Palatinose
D1 D-Raffinose
D2 Salicin
D3 Sedoheptulosan
D4 L-Sorbose
D5 Stachyose
D6 D-Tagatose
D7 Turanose
D8 Xylitol
D9 N-Acetyl-D Glucosaminitol
D10 γ -Amino Butyric Acid
D11 δ -Amino Valeric Acid
D12 Butyric Acid
E1 Capric Acid
E2 Caproic Acid
E3 Citraconic Acid
E4 Citramalic Acid
E5 D-Glucosamine
E6 2-Hydroxy Benzoic Acid
E7 4-Hydroxy Benzoic Acid
E8 β -Hydroxy Butyric Acid
E9 γ -Hydroxy Butyric Acid
E10 α -Keto Valeric Acid E11 Itaconic Acid
E12 5-Keto-DGluconic Acid
F1 D-Lactic Acid Methyl Ester
F2 Malonic Acid
F3 Melibionc Acid
F4 Oxalic Acid
F5 Oxalomalic Acid
F6 Quinic Acid

F7 D-Ribono-1,4Lactone
F8 Sebacic Acid
F9 Sorbic Acid
F10 Succinamic Acid
F11 D-Tartaric Acid
F12 L-Tartaric Acid
G1 Acetamide
G2 L-Alaninamide
G3 N-Acetyl-LGlutamic Acid
G4 L-Arginine
G5 Glycine
G6 L-Histidine
G7 L-Homoserine
G8 Hydroxy-LProline
G9 L-Isoleucine
G10 L-Leucine
G11 L-Lysine
G12 L-Methionine
H1 L-Ornithine
H2 L Phenylalanine
H3 L-Pyroglutamic Acid
H4 L-Valine
H5 D,L-Carnitine
H6 Sec-Butylamine
H7 D.LOctopamine
H8 Putrescine
H9 Dihydroxy Acetone
H10 2,3-Butanediol
H11 2,3-Butanone
H12 3-Hydroxy 2Butanone

PM10 MicroPlate™ pH Sources

A1 pH 3.5

A2 pH 4

A3 pH 4.5

A4 pH 5

A5 pH 5.5

A6 pH 6

A7 pH 7

A8 pH 8

A9 pH 8.5

A10 pH 9

A11 pH 9.5

A12 pH 10

B1 pH 4.5

B2 pH 4.5 + L-Alanine

B3 pH 4.5 + L-Arginine

B4 pH 4.5 + L-Asparagine

B5 pH 4.5 + L-Aspartic Acid

B6 pH 4.5 + L-Glutamic Acid

B7 pH 4.5 + L-Glutamine

B8 pH 4.5 + Glycine

B9 pH 4.5 + L-Histidine

B10 pH 4.5 + L-Isoleucine

B11 pH 4.5 + L-Leucine

B12 pH 4.5 + L-Lysine

C1 pH 4.5 + L-Methionine

C2 pH 4.5 + L-Phenylalanine

C3 pH 4.5 + L-Proline

C4 pH 4.5 + L-Serine

C5 pH 4.5 + L-Threonine

C6 pH 4.5 + L-Tryptophan

C7 pH 4.5 + L-Tyrosine

C8 pH 4.5 + L-Valine

C9 pH 4.5 + Hydroxy- L-Proline
C10 pH 4.5 + L-Ornithine
C11 pH 4.5 + L-Homoarginine
C12 pH 4.5 + L-Homoserine
D-1 pH 4.5 + Anthranilic acid
D2 pH 4.5 + L-Norleucine
D3 pH 4.5 + L-Norvaline
D4 pH 4.5 + α - Amino-Nbutyric acid
D5 pH 4.5 + pAminobenzoate
D6 pH 4.5 + L-Cysteic acid
D7 pH 4.5 + D-Lysine
D8 pH 4.5 + 5-Hydroxy Lysine
D9 pH 4.5 + 5-Hydroxy Tryptophan
D10 pH 4.5 + D,L-Diamino pimelic acid
D11 pH 4.5 + Trimethyl amine-N-oxide
D12 pH 4.5 + Urea
E1 pH 9.5
E2 pH 9.5 + L-Alanine
E3 pH 9.5 + L-Arginine
E4 pH 9.5 + L-Asparagine
E5 pH 9.5 + L-Aspartic Acid
E6 pH 9.5 + L-Glutamic Acid
E7 pH 9.5 + L-Glutamine
E8 pH 9.5 + Glycine
E9 pH 9.5 + L-Histidine
E10 pH 9.5 + L-Isoleucine
E11 pH 9.5 + L-Leucine
E12 pH 9.5 + L-Lysine
F1 pH 9.5 + L-Methionine
F2 pH 9.5 + LPhenylalanine
F3 pH 9.5 + L-Proline
F4 pH 9.5 + L-Serine
F5 pH 9.5 + L-Threonine
F6 pH 9.5 + L-Tryptophan

F7 pH 9.5 + L-Tyrosine
F8 pH 9.5 + L-Valine
F9 pH 9.5 + Hydroxy- L-Proline
F10 pH 9.5 + L-Ornithine
F11 pH 9.5 + L-Homoarginine
F12 pH 9.5 + L-Homoserine
G1 pH 9.5 + Anthranilic acid
G2 pH 9.5 + L-Norleucine
G3 pH 9.5 + L-Norvaline
G4 pH 9.5 + Agmatine
G5 pH 9.5 + Cadaverine
G6 pH 9.5 + Putrescine
G7 pH 9.5 + Histamine
G8 pH 9.5 + Phenylethylamine
G9 pH 9.5 + Tyramine
G10 pH 9.5 + Creatine
G11 pH 9.5 + Trimethyl amine-N-oxide
G12 pH 9.5 + Urea
H1 X-Caprylate
H2 X- α -D- Glucoside
H3 X- β -D- Glucoside
H4 X- α -D-Galactoside
H5 X- β -D- Galactoside
H6 X- α - DGlucuronide
H7 X- β - D- Glucuronide
H8 X- β -DGlucosaminide
H9 X- β -DGalactosaminide
H10 X- α -D- Mannoside
H11 X-PO₄
H12 X-SO₄

PM11C MicroPlate™

A1 Amikacin 1
A2 Amikacin 2
A3 Amikacin 3
A4 Amikacin 4
A5 Chlortetracycline 1
A6 Chlortetracycline 2
A7 Chlortetracycline 3
A8 Chlortetracycline 4
A9 Lincomycin 1
A10 Lincomycin 2
A11 Lincomycin 3
A12 Lincomycin 4
B1 Amoxicillin 1
B2 Amoxicillin 2
B3 Amoxicillin 3
B4 Amoxicillin 4
B5 Cloxacillin 1
B6 Cloxacillin 2
B7 Cloxacillin 3
B8 Cloxacillin 4
B9 Lomefloxacin 1
B10 Lomefloxacin 2
B11 Lomefloxacin 3
B12 Lomefloxacin 4
C1 Bleomycin 1
C2 Bleomycin 2
C3 Bleomycin 3
C4 Bleomycin 4
C5 Colistin 1
C6 Colistin 2
C7 Colistin 3
C8 Colistin 4
C9 Minocycline 1

C10 Minocycline 2
C11 Minocycline 3
C12 Minocycline 4
D1 Capreomycin 1
D2 Capreomycin 2
D3 Capreomycin 3
D4 Capreomycin 4
D5 Demeclocycline 1
D6 Demeclocycline 2
D7 Demeclocycline 3
D8 Demeclocycline 4
D9 Nafcillin 1
D10 Nafcillin 2
D11 Nafcillin 3
D12 Nafcillin 4
E1 Cefazolin 1
E2 Cefazolin 2
E3 Cefazolin 3
E4 Cefazolin 4
E5 Enoxacin 1
E6 Enoxacin 2
E7 Enoxacin 3
E8 Enoxacin 4
E9 Nalidixic acid 1
E10 Nalidixic acid 2
E11 Nalidixic acid 3
E12 Nalidixic acid 4
F1 Chloramphenicol 1
F2 Chloramphenicol 2
F3 Chloramphenicol 3
F4 Chloramphenicol 4
F5 Erythromycin 1
F6 Erythromycin 2
F7 Erythromycin 3

F8 Erythromycin 4
F9 Neomycin 1
F10 Neomycin 2
F11 Neomycin 3
F12 Neomycin 4
G1 Ceftriaxone 1
G2 Ceftriaxone 2
G3 Ceftriaxone 3
G4 Ceftriaxone 4
G5 Gentamicin 1
G6 Gentamicin 2
G7 Gentamicin 3
G8 Gentamicin 4
G9 Potassium tellurite 1
G10 Potassium tellurite 2
G11 Potassium tellurite 3
G12 Potassium tellurite 4
H1 Cephalothin 1
H2 Cephalothin 2
H3 Cephalothin 3
H4 Cephalothin 4
H5 Kanamycin 1
H6 Kanamycin 2
H7 Kanamycin 3
H8 Kanamycin 4
H9 Ofloxacin 1
H10 Ofloxacin 2
H11 Ofloxacin 3
H12 Ofloxacin 4

PM12B MicroPlate™

A1 Penicillin G 1
A2 Penicillin G 2
A3 Penicillin G 3
A4 Penicillin G 4
A5 Tetracycline 1
A6 Tetracycline 2
A7 Tetracycline 3
A8 Tetracycline 4
A9 Carbenicillin 1
A10 Carbenicillin 2
A11 Carbenicillin 3
A12 Carbenicillin 4
B1 Oxacillin 1
B2 Oxacillin 2
B3 Oxacillin 3
B4 Oxacillin 4
B5 Penimepicycline 1
B6 Penimepicycline 2
B7 Penimepicycline 3
B8 Penimepicycline 4
B9 Polymyxin B 1
B10 Polymyxin B 2
B11 Polymyxin B 3
B12 Polymyxin B 4
C1 Paromomycin 1
C2 Paromomycin 2
C3 Paromomycin 3
C4 Paromomycin 4
C5 Vancomycin 1
C6 Vancomycin 2
C7 Vancomycin 3
C8 Vancomycin 4
C9 D,L-Serine hydroxamate 1

C10 D,L-Serine hydroxamate 2
C11 D,L-Serine hydroxamate 3
C12 D,L-Serine hydroxamate 4
D1 Sisomicin 1
D2 Sisomicin 2
D3 Sisomicin 3
D4 Sisomicin 4
D5 Sulfamethazine 1
D6 Sulfamethazine 2
D7 Sulfamethazine 3
D8 Sulfamethazine 4
D9 Novobiocin 1
D10 Novobiocin 2
D11 Novobiocin 3
D12 Novobiocin 4
E1 2,4-Diamino-6,7diisopropylpteridine 1
E2 2,4-Diamino-6,7diisopropylpteridine 2
E3 2,4-Diamino-6,7diisopropylpteridine 3
E4 2,4-Diamino-6,7diisopropylpteridine 4
E5 Sulfadiazine 1
E6 Sulfadiazine 2
E7 Sulfadiazine 3
E8 Sulfadiazine 4
E9 Benzethonium chloride 1
E10 Benzethonium chloride 2
E11 Benzethonium chloride 3
E12 Benzethonium chloride 4
F1 Tobramycin 1
F2 Tobramycin 2
F3 Tobramycin 3
F4 Tobramycin 4
F5 Sulfathiazole 1
F6 Sulfathiazole 2
F7 Sulfathiazole 3

F8 Sulfathiazole 4
F9 5-Fluoroorotic acid 1
F10 5-Fluoroorotic acid 2
F11 5-Fluoroorotic acid 3
F12 5-Fluoroorotic acid 4
G1 Spectinomycin 1
G2 Spectinomycin 2
G3 Spectinomycin 3
G4 Spectinomycin 4
G5 Sulfamethoxazole 1
G6 Sulfamethoxazole 2
G7 Sulfamethoxazole 3
G8 Sulfamethoxazole 4
G9 L- Aspartic - β - hydroxamate 1
G10 L- Aspartic - β - hydroxamate 2
G11 L- Aspartic - β - hydroxamate 3
G12 L- Aspartic - β - hydroxamate 4
H1 Spiramycin 1
H2 Spiramycin 2
H3 Spiramycin 3
H4 Spiramycin 4
H5 Rifampicin 1
H6 Rifampicin 2
H7 Rifampicin 3
H8 Rifampicin 4
H9 Dodecyltrimethyl ammonium bromide 1
H10 Dodecyltrimethyl ammonium bromide 2
H11 Dodecyltrimethyl ammonium bromide 3
H12 Dodecyltrimethyl ammonium bromide 4

PM13B MicroPlate™

A1 Ampicillin 1

A2 Ampicillin 2

A3 Ampicillin 3

A4 Ampicillin 4

A5 Dequalinium chloride 1

A6 Dequalinium chloride 2

A7 Dequalinium chloride 3

A8 Dequalinium chloride 4

A9 Nickel chloride 1

A10 Nickel chloride 2

A11 Nickel chloride 3

A12 Nickel chloride 4

B1 Azlocillin 1

B2 Azlocillin 2

B3 Azlocillin 3

B4 Azlocillin 4

B5 2,2'-Dipyridyl 1

B6 2,2'-Dipyridyl 2

B7 2,2'-Dipyridyl 3

B8 2,2'-Dipyridyl 4

B9 Oxolinic acid 1

B10 Oxolinic acid 2

B11 Oxolinic acid 3

B12 Oxolinic acid 4

C1 6-Mercaptopurine 1

C2 6-Mercaptopurine 2

C3 6-Mercaptopurine 3

C4 6-Mercaptopurine 4

C5 Doxycycline 1

C6 Doxycycline 2

C7 Doxycycline 3

C8 Doxycycline 4

C9 Potassium chromate 1

C10 Potassium chromate 2
C11 Potassium chromate 3
C12 Potassium chromate 4
D1 Cefuroxime 1
D2 Cefuroxime 2
D3 Cefuroxime 3
D4 Cefuroxime 4
D5 5-Fluorouracil 1
D6 5-Fluorouracil 2
D7 5-Fluorouracil 3
D8 5-Fluorouracil 4
D9 Rolitetracycline 1
D10 Rolitetracycline 2
D11 Rolitetracycline 3
D12 Rolitetracycline 4
E1 Cytosine arabinoside 1
E2 Cytosine arabinoside 2
E3 Cytosine arabinoside 3
E4 Cytosine arabinoside 4
E5 Geneticin (G418) 1
E6 Geneticin (G418) 2
E7 Geneticin (G418) 3
E8 Geneticin (G418) 4
E9 Ruthenium red 1
E10 Ruthenium red 2
E11 Ruthenium red 3
E12 Ruthenium red 4
F1 Cesium chloride 1
F2 Cesium chloride 2
F3 Cesium chloride 3
F4 Cesium chloride 4
F5 Glycine 1
F6 Glycine 2
F7 Glycine 3

F8 Glycine 4
F9 Thallium (I) acetate 1
F10 Thallium (I) acetate 2
F11 Thallium (I) acetate 3
F12 Thallium (I) acetate 4
G1 Cobalt chloride 1
G2 Cobalt chloride 2
G3 Cobalt chloride 3
G4 Cobalt chloride 4
G5 Manganese (II) chloride 1
G6 Manganese (II) chloride 2
G7 Manganese (II) chloride 3
G8 Manganese (II) chloride 4
G9 Trifluoperazine 1
G10 Trifluoperazine 2
G11 Trifluoperazine 3
G12 Trifluoperazine 4
H1 Cupric chloride 1
H2 Cupric chloride 2
H3 Cupric chloride 3
H4 Cupric chloride 4
H5 Moxalactam 1
H6 Moxalactam 2
H7 Moxalactam 3
H8 Moxalactam 4
H9 Tylosin 1
H10 Tylosin 2
H11 Tylosin 3
H12 Tylosin 4

PM14A MicroPlate™

A1 Acriflavine 1

A2 Acriflavine 2

A3 Acriflavine 3

A4 Acriflavine 4

A5 Furaltadone 1

A6 Furaltadone 2

A7 Furaltadone 3

A8 Furaltadone 4

A9 Sanguinarine 1

A10 Sanguinarine 2

A11 Sanguinarine 3

A12 Sanguinarine 4

B1 9-Aminoacridine 1

B2 9-Aminoacridine 2

B3 9-Aminoacridine 3

B4 9-Aminoacridine 4

B5 Fusaric acid 1

B6 Fusaric acid 2

B7 Fusaric acid 3

B8 Fusaric acid 4

B9 Sodium arsenate 1

B10 Sodium arsenate 2

B11 Sodium arsenate 3

B12 Sodium arsenate 4

C1 Boric Acid 1

C2 Boric Acid 2

C3 Boric Acid 3

C4 Boric Acid 4

C5 1-Hydroxypyridine2-thione 1

C6 1-Hydroxypyridine2-thione 2

C7 1-Hydroxypyridine2-thione 3

C8 1-Hydroxypyridine2-thione 4

C9 Sodium cyanate 1

C10 Sodium cyanate 2
C11 Sodium cyanate 3
C12 Sodium cyanate 4
D1 Cadmium chloride 1
D2 Cadmium chloride 2
D3 Cadmium chloride 3
D4 Cadmium chloride 4
D5 Iodoacetate 1
D6 Iodoacetate 2
D7 Iodoacetate 3
D8 Iodoacetate 4
D9 Sodium dichromate 1
D10 Sodium dichromate 2
D11 Sodium dichromate 3
D12 Sodium dichromate 4
E1 Cefoxitin 1
E2 Cefoxitin 2
E3 Cefoxitin 3
E4 Cefoxitin 4
E5 Nitrofurantoin 1
E6 Nitrofurantoin 2
E7 Nitrofurantoin 3
E8 Nitrofurantoin 4
E9 Sodium metaborate 1
E10 Sodium metaborate 2
E11 Sodium metaborate 3
E12 Sodium metaborate 4
F1 Chloramphenicol 1
F2 Chloramphenicol 2
F3 Chloramphenicol 3
F4 Chloramphenicol 4
F5 Piperacillin 1
F6 Piperacillin 2
F7 Piperacillin 3

F8 Piperacillin 4
F9 Sodium metavanadate 1
F10 Sodium metavanadate 2
F11 Sodium metavanadate 3
F12 Sodium metavanadate 4
G1 Chelerythrine 1
G2 Chelerythrine 2
G3 Chelerythrine 3
G4 Chelerythrine 4
G5 Carbenicillin 1
G6 Carbenicillin 2
G7 Carbenicillin 3
G8 Carbenicillin 4
G9 Sodium Nitrite 1
G10 Sodium Nitrite 2
G11 Sodium Nitrite 3
G12 Sodium Nitrite 4
H1 EGTA 1
H2 EGTA 2
H3 EGTA 3
H4 EGTA 4
H5 Promethazine 1
H6 Promethazine 2
H7 Promethazine 3
H8 Promethazine 4
H9 Sodium orthovanadate 1
H10 Sodium orthovanadate 2
H11 Sodium orthovanadate 3
H12 Sodium orthovanadate 4

PM15B MicroPlate™

A1 Procaine 1

A2 Procaine 2

A3 Procaine 3

A4 Procaine 4

A5 Guanidine hydrochloride 1

A6 Guanidine hydrochloride 2

A7 Guanidine hydrochloride 3

A8 Guanidine hydrochloride 4

A9 Cefmetazole 1

A10 Cefmetazole 2

A11 Cefmetazole 3

A12 Cefmetazole 4

B1 D-Cycloserine 1

B2 D-Cycloserine 2

B3 D-Cycloserine 3

B4 D-Cycloserine 4

B5 EDTA 1

B6 EDTA 2

B7 EDTA 3

B8 EDTA 4

B9 5,7-Dichloro-8hydroxy-quinaldine 1

B10 5,7-Dichloro-8hydroxy-quinaldine 2

B11 5,7-Dichloro-8hydroxy-quinaldine 3

B12 5,7-Dichloro-8hydroxy-quinaldine 4

C1 5,7-Dichloro-8hydroxyquinoline 1

C2 5,7-Dichloro-8hydroxyquinoline 2

C3 5,7-Dichloro-8hydroxyquinoline 3

C4 5,7-Dichloro-8hydroxyquinoline 4

C5 Fusidic acid 1

C6 Fusidic acid 2

C7 Fusidic acid 3

C8 Fusidic acid 4

C9 1,10Phenanthroline 1

C10 1,10Phenanthroline 2
C11 1,10Phenanthroline 3
C12 1,10Phenanthroline 4
D1 Phleomycin 1
D2 Phleomycin 2
D3 Phleomycin 3
D4 Phleomycin 4
D5 Domiphen bromide 1
D6 Domiphen bromide 2
D7 Domiphen bromide 3
D8 Domiphen bromide 4
D9 Nordihydroguaia retic acid 1
D10 Nordihydroguaia retic acid 2
D11 Nordihydroguaia retic acid 3
D12 Nordihydroguaia retic acid 4
E1 Alexidine 1
E2 Alexidine 2
E3 Alexidine 3
E4 Alexidine 4
E5 Nitrofurazone 1
E6 Nitrofurazone 2
E7 Nitrofurazone 3
E8 Nitrofurazone 4
E9 Methyl viologen 1
E10 Methyl viologen 2
E11 Methyl viologen 3
E12 Methyl viologen 4
F1 3, 4Dimethoxybenzyl alcohol 1
F2 3, 4Dimethoxybenzyl alcohol 2
F3 3, 4Dimethoxybenzyl alcohol 3
F4 3, 4Dimethoxybenzyl alcohol 4
F5 Oleandomycin 1
F6 Oleandomycin 2
F7 Oleandomycin 3

F8 Oleandomycin 4
F9 Puromycin 1
F10 Puromycin 2
F11 Puromycin 3
F12 Puromycin 4
G1 CCCP 1
G2 CCCP 2
G3 CCCP 3
G4 CCCP 4
G5 Sodium azide 1
G6 Sodium azide 2
G7 Sodium azide 3
G8 Sodium azide 4
G9 Menadione 1
G10 Menadione 2
G11 Menadione 3
G12 Menadione 4
H1 2-Nitroimidazole 1
H2 2-Nitroimidazole 2
H3 2-Nitroimidazole 3
H4 2-Nitroimidazole 4
H5 Hydroxyurea 1
H6 Hydroxyurea 2
H7 Hydroxyurea 3
H8 Hydroxyurea 4
H9 Zinc chloride 1
H10 Zinc chloride 2
H11 Zinc chloride 3
H12 Zinc chloride 4

PM16A MicroPlate™

A1 Cefotaxime 1
A2 Cefotaxime 2
A3 Cefotaxime 3
A4 Cefotaxime 4
A5 Phosphomycin 1
A6 Phosphomycin 2
A7 Phosphomycin 3
A8 Phosphomycin 4
A9 5-Chloro-7-iodo-8hydroxyquinoline 1
A10 5-Chloro-7-iodo-8hydroxyquinoline 2
A11 5-Chloro-7-iodo-8hydroxyquinoline 3
A12 5-Chloro-7-iodo-8hydroxyquinoline 4
B1 Norfloxacin 1
B2 Norfloxacin 2
B3 Norfloxacin 3
B4 Norfloxacin 4
B5 Sulfanilamide 1
B6 Sulfanilamide 2
B7 Sulfanilamide 3
B8 Sulfanilamide 4
B9 Trimethoprim 1
B10 Trimethoprim 2
B11 Trimethoprim 3
B12 Trimethoprim 4
C1 Dichlofluanid 1
C2 Dichlofluanid 2
C3 Dichlofluanid 3
C4 Dichlofluanid 4
C5 Protamine sulfate 1
C6 Protamine sulfate 2
C7 Protamine sulfate 3
C8 Protamine sulfate 4
C9 Cetylpyridinium chloride 1

C10 Cetylpyridinium chloride 2
C11 Cetylpyridinium chloride 3
C12 Cetylpyridinium chloride 4
D1 1-Chloro-2,4dinitrobenzene 1
D2 1-Chloro-2,4dinitrobenzene 2
D3 1-Chloro-2,4dinitrobenzene 3
D4 1-Chloro-2,4dinitrobenzene 4
D5 Diamide 1
D6 Diamide 2
D7 Diamide 3
D8 Diamide 4
D9 Cinoxacin 1
D10 Cinoxacin 2
D11 Cinoxacin 3
D12 Cinoxacin 4
E1 Streptomycin 1
E2 Streptomycin 2
E3 Streptomycin 3
E4 Streptomycin 4
E5 5-Azacytidine 1
E6 5-Azacytidine 2
E7 5-Azacytidine 3
E8 5-Azacytidine 4
E9 Rifamycin SV 1
E10 Rifamycin SV 2
E11 Rifamycin SV 3
E12 Rifamycin SV 4
F1 Potassium tellurite 1
F2 Potassium tellurite 2
F3 Potassium tellurite 3
F4 Potassium tellurite 4
F5 Sodium selenite 1
F6 Sodium selenite 2
F7 Sodium selenite 3

F8 Sodium selenite 4
F9 Aluminum sulfate 1
F10 Aluminum sulfate 2
F11 Aluminum sulfate 3
F12 Aluminum sulfate 4
G1 Chromium chloride 1
G2 Chromium chloride 2
G3 Chromium chloride 3
G4 Chromium chloride 4
G5 Ferric chloride 1
G6 Ferric chloride 2
G7 Ferric chloride 3
G8 Ferric chloride 4
G9 L-Glutamic acid g-hydroxamate 1
G10 L-Glutamic acid g-hydroxamate 2
G11 L-Glutamic acid g-hydroxamate 3
G12 L-Glutamic acid g-hydroxamate 4
H1 Glycine hydroxamate 1
H2 Glycine hydroxamate 2
H3 Glycine hydroxamate 3
H4 Glycine hydroxamate 4
H5 Chloroxylenol 1
H6 Chloroxylenol 2
H7 Chloroxylenol 3
H8 Chloroxylenol 4
H9 Sorbic Acid 1
H10 Sorbic Acid 2
H11 Sorbic Acid 3
H12 Sorbic Acid 4

PM17A MicroPlate™

A1 D-Serine 1

A2 D-Serine 2

A3 D-Serine 3

A4 D-Serine 4

A5 β -Chloro-L-alanine 1

A6 β -Chloro-L-alanine 2

A7 β -Chloro-L-alanine 3

A8 β -Chloro-L-alanine 4

A9 Thiosalicylate 1

A10 Thiosalicylate 2

A11 Thiosalicylate 3

A12 Thiosalicylate 4

B1 Sodium salicylate 1

B2 Sodium salicylate 2

B3 Sodium salicylate 3

B4 Sodium salicylate 4

B5 Hygromycin B 1

B6 Hygromycin B 2

B7 Hygromycin B 3

B8 Hygromycin B 4

B9 Ethionamide 1

B10 Ethionamide 2

B11 Ethionamide 3

B12 Ethionamide 4

C1 4-Aminopyridine 1

C2 4-Aminopyridine 2

C3 4-Aminopyridine 3

C4 4-Aminopyridine 4

C5 Sulfachloropyridazine 1

C6 Sulfachloropyridazine 2

C7 Sulfachloropyridazine 3

C8 Sulfachloropyridazine 4

C9 Sulfamonomethoxine 1

C10 Sulfamonomethoxine 2
C11 Sulfamonomethoxine 3
C12 Sulfamonomethoxine 4
D1 Oxycarboxin 1
D2 Oxycarboxin 2
D3 Oxycarboxin 3
D4 Oxycarboxin 4
D5 Aminotriazole 1
D6 Aminotriazole 2
D7 Aminotriazole 3
D8 Aminotriazole 4
D9 Chlorpromazine 1
D10 Chlorpromazine 2
D11 Chlorpromazine 3
D12 Chlorpromazine 4
E1 Niaproof 1
E2 Niaproof 2
E3 Niaproof 3
E4 Niaproof 4
E5 Compound 48/80 1
E6 Compound 48/80 2
E7 Compound 48/80 3
E8 Compound 48/80 4
E9 Sodium tungstate 1
E10 Sodium tungstate 2
E11 Sodium tungstate 3
E12 Sodium tungstate 4
F1 Lithium chloride 1
F2 Lithium chloride 2
F3 Lithium chloride 3
F4 Lithium chloride 4
F5 D,L-Methionine hydroxamate 1
F6 D,L-Methionine hydroxamate 2
F7 D,L-Methionine hydroxamate 3

F8 D,L-Methionine hydroxamate 4
F9 Tannic acid 1
F10 Tannic acid 2
F11 Tannic acid 3
F12 Tannic acid 4
G1 Chlorambucil 1
G2 Chlorambucil 2
G3 Chlorambucil 3
G4 Chlorambucil 4
G5 Cefamandole 1
G6 Cefamandole 2
G7 Cefamandole 3
G8 Cefamandole 4
G9 Cefoperazone 1
G10 Cefoperazone 2
G11 Cefoperazone 3
G12 Cefoperazone 4
H1 Cefsulodin 1
H2 Cefsulodin 2
H3 Cefsulodin 3
H4 Cefsulodin 4
H5 Caffeine 1
H6 Caffeine 2
H7 Caffeine 3
H8 Caffeine 4
H9 Phenylarsine oxide 1
H10 Phenylarsine oxide 2
H11 Phenylarsine oxide 3
H12 Phenylarsine oxide 4

PM18C MicroPlate™

A1 Ketoprofen 1
A2 Ketoprofen 2
A3 Ketoprofen 3
A4 Ketoprofen 4
A5 Pyrophosphate 1
A6 Pyrophosphate 2
A7 Pyrophosphate 3
A8 Pyrophosphate 4
A9 Thiamphenicol 1
A10 Thiamphenicol 2
A11 Thiamphenicol 3
A12 Thiamphenicol 4
B1 Trifluorothymidine 1
B2 Trifluorothymidine 2
B3 Trifluorothymidine 3
B4 Trifluorothymidine 4
B5 Pipemidic Acid 1
B6 Pipemidic Acid 2
B7 Pipemidic Acid 3
B8 Pipemidic Acid 4
B9 Azathioprine 1
B10 Azathioprine 2
B11 Azathioprine 3
B12 Azathioprine 4
C1 Poly-L-lysine 1
C2 Poly-L-lysine 2
C3 Poly-L-lysine 3
C4 Poly-L-lysine 4
C5 Sulfisoxazole 1
C6 Sulfisoxazole 2
C7 Sulfisoxazole 3
C8 Sulfisoxazole 4
C9 Pentachlorophenol 1

C10 Pentachlorophenol 2
C11 Pentachlorophenol 3
C12 Pentachlorophenol 4
D1 Sodium m-arsenite 1
D2 Sodium m-arsenite 2
D3 Sodium m-arsenite 3
D4 Sodium m-arsenite 4
D5 Sodium bromate 1
D6 Sodium bromate 2
D7 Sodium bromate 3
D8 Sodium bromate 4
D9 Lidocaine 1
D10 Lidocaine 2
D11 Lidocaine 3
D12 Lidocaine 4
E1 Sodium metasilicate 1
E2 Sodium metasilicate 2
E3 Sodium metasilicate 3
E4 Sodium metasilicate 4
E5 Sodium periodate 1
E6 Sodium periodate 2
E7 Sodium periodate 3
E8 Sodium periodate 4
E9 Antimony (III) chloride 1
E10 Antimony (III) chloride 2
E11 Antimony (III) chloride 3
E12 Antimony (III) chloride 4
F1 Semicarbazide hydrochloride 1
F2 Semicarbazide hydrochloride 2
F3 Semicarbazide hydrochloride 3
F4 Semicarbazide hydrochloride 4
F5 Tinidazole 1
F6 Tinidazole 2
F7 Tinidazole 3

F8 Tinidazole 4
F9 Aztreonam 1
F10 Aztreonam 2
F11 Aztreonam 3
F12 Aztreonam 4
G1 Triclosan 1
G2 Triclosan 2
G3 Triclosan 3
G4 Triclosan 4
G5 Guanazole 1
G6 Guanazole 2
G7 Guanazole 3
G8 Guanazole 4
G9 Myricetin 1
G10 Myricetin 2
G11 Myricetin 3
G12 Myricetin 4
H1 5-Fluoro-5'deoxyuridine 1
H2 5-Fluoro-5'deoxyuridine 2
H3 5-Fluoro-5'deoxyuridine 3
H4 5-Fluoro-5'deoxyuridine 4
H5 2-Phenylphenol 1
H6 2-Phenylphenol 2
H7 2-Phenylphenol 3
H8 2-Phenylphenol 4
H9 Plumbagin 1
H10 Plumbagin 2
H11 Plumbagin 3
H12 Plumbagin 4

PM19 MicroPlate™

A1 Josamycin 1

A2 Josamycin 2

A3 Josamycin 3

A4 Josamycin 4

A5 Gallic acid 1

A6 Gallic acid 2

A7 Gallic acid 3

A8 Gallic acid 4

A9 Coumarin 1

A10 Coumarin 2

A11 Coumarin 3

A12 Coumarin 4

B1 Methyltrioctylammonium chloride 1

B2 Methyltrioctylammonium chloride 2

B3 Methyltrioctylammonium chloride 3

B4 Methyltrioctylammonium chloride 4

B5 Harmane 1

B6 Harmane 2

B7 Harmane 3

B8 Harmane 4

B9 2,4-Dinitrophenol 1

B10 2,4-Dinitrophenol 2

B11 2,4-Dinitrophenol 3

B12 2,4-Dinitrophenol 4

C1 Chlorhexidine 1

C2 Chlorhexidine 2

C3 Chlorhexidine 3

C4 Chlorhexidine 4

C5 Umbelliferone 1

C6 Umbelliferone 2

C7 Umbelliferone 3

C8 Umbelliferone 4

C9 Cinnamic acid 1

C10 Cinnamic acid 2
C11 Cinnamic acid 3
C12 Cinnamic acid 4
D1 Disulfiram 1
D2 Disulfiram 2
D3 Disulfiram 3
D4 Disulfiram 4
D5 Iodonitro tetrazolium violet 1
D6 Iodonitro tetrazolium violet 2
D7 Iodonitro tetrazolium violet 3
D8 Iodonitro tetrazolium violet 4
D9 Phenylmethanesulfonyl fluoride (PMSF) 1
D10 Phenylmethanesulfonyl fluoride (PMSF) 2
D11 Phenylmethanesulfonyl fluoride (PMSF) 3
D12 Phenylmethanesulfonyl fluoride (PMSF) 4
E1 FCCP 1
E2 FCCP 2
E3 FCCP 3
E4 FCCP 4
E5 D,L-Thioctic acid 1
E6 D,L-Thioctic acid 2
E7 D,L-Thioctic acid 3
E8 D,L-Thioctic acid 4
E9 Lawsone 1
E10 Lawsone 2
E11 Lawsone 3
E12 Lawsone 4
F1 Phenethicillin 1
F2 Phenethicillin 2
F3 Phenethicillin 3
F4 Phenethicillin 4
F5 Blastidicin S 1
F6 Blastidicin S 2
F7 Blastidicin S 3

F8 Blasticidin S 4
F9 Sodium caprylate 1
F10 Sodium caprylate 2
F11 Sodium caprylate 3
F12 Sodium caprylate 4
G1 Lauryl sulfobetaine 1
G2 Lauryl sulfobetaine 2
G3 Lauryl sulfobetaine 3
G4 Lauryl sulfobetaine 4
G5 Dihydrostreptomycin 1
G6 Dihydrostreptomycin 2
G7 Dihydrostreptomycin 3
G8 Dihydro- streptomycin 4
G9 Hydroxylamine 1
G10 Hydroxylamine 2
G11 Hydroxylamine 3
G12 Hydroxylamine 4
H1 Hexamminecobalt (III) Chloride 1
H2 Hexamminecobalt (III) Chloride 2
H3 Hexamminecobalt (III) Chloride 3
H4 Hexamminecobalt (III) Chloride 4
H5 Thioglycerol 1
H6 Thioglycerol 2
H7 Thioglycerol 3
H8 Thioglycerol 4
H9 Polymyxin B 1
H10 Polymyxin B 2
H11 Polymyxin B 3
H12 Polymyxin B 4

PM20B MicroPlate™

A1 Amitriptyline 1

A2 Amitriptyline 2

A3 Amitriptyline 3

A4 Amitriptyline 4

A5 Apramycin 1

A6 Apramycin 2

A7 Apramycin 3

A8 Apramycin 4

A9 Benserazide 1

A10 Benserazide 2

A11 Benserazide 3

A12 Benserazide 4

B1 Orphenadrine 1

B2 Orphenadrine 2

B3 Orphenadrine 3

B4 Orphenadrine 4

B5 Propranolol 1

B6 Propranolol 2

B7 Propranolol 3

B8 Propranolol 4

B9 Tetrazolium violet 1

B10 Tetrazolium violet 2

B11 Tetrazolium violet 3

B12 Tetrazolium violet 4

C1 Thioridazine 1

C2 Thioridazine 2

C3 Thioridazine 3

C4 Thioridazine 4

C5 Atropine 1

C6 Atropine 2

C7 Atropine 3

C8 Atropine 4

C9 Ornidazole 1

C10 Ornidazole 2
C11 Ornidazole 3
C12 Ornidazole 4
D1 Proflavine 1
D2 Proflavine 2
D3 Proflavine 3
D4 Proflavine 4
D5 Ciprofloxacin 1
D6 Ciprofloxacin 2
D7 Ciprofloxacin 3
D8 Ciprofloxacin 4
D9 18-Crown-6 ether 1
D10 18-Crown-6 ether 2
D11 18-Crown-6 ether 3
D12 18-Crown-6 ether 4
E1 Crystal Violet 1
E2 Crystal Violet 2
E3 Crystal Violet 3
E4 Crystal Violet 4
E5 Dodine 1
E6 Dodine 2
E7 Dodine 3
E8 Dodine 4
E9 Hexachlorophene 1
E10 Hexachlorophene 2
E11 Hexachlorophene 3
E12 Hexachlorophene 4
F1 4Hydroxycoumarin 1
F2 4Hydroxycoumarin 2
F3 4Hydroxycoumarin 3
F4 4Hydroxycoumarin 4
F5 Oxytetracycline 1
F6 Oxytetracycline 2
F7 Oxytetracycline 3

F8 Oxytetracycline 4
F9 Pridinol 1
F10 Pridinol 2
F11 Pridinol 3
F12 Pridinol 4
G1 Captan 1
G2 Captan 2
G3 Captan 3
G4 Captan 4
G5 3, 5-Dinitrobenzene 1
G6 3, 5-Dinitrobenzene 2
G7 3, 5-Dinitrobenzene 3
G8 3,5Dinitrobenzene 4
G9 8Hydroxyquinoline 1
G10 8Hydroxyquinoline 2
G11 8Hydroxyquinoline 3
G12 8Hydroxyquinoline 4
H1 Patulin 1
H2 Patulin 2
H3 Patulin 3
H4 Patulin 4
H5 Tolyfluanid 1
H6 Tolyfluanid 2
H7 Tolyfluanid 3
H8 Tolyfluanid 4
H9 Troleandomycin 1
H10 Troleandomycin 2
H11 Troleandomycin 3
H12 Troleandomycin 4